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GRANT NUMBER DAMD17-96-1-6253

TITLE: Targeting HER-2/neu Overexpression By Suicide Ribozyme In Breast Cancer

PRINCIPAL INVESTIGATOR: Mien-Chie Hung, Ph.D.

CONTRACTING ORGANIZATION: University of Texas
M. D. Anderson Cancer Center
Houston, Texas 77030

REPORT DATE: September 1998

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

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19990415036

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE September 1998	3. REPORT TYPE AND DATES COVERED Final (1 Sep 96 - 31 Aug 98)	
4. TITLE AND SUBTITLE Targeting HER-2/neu Overexpression By Suicide Ribozyme In Breast Cancer			5. FUNDING NUMBERS DAMD17-96-1-6253	
6. AUTHOR(S) Mien-Chie Hung, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The University of Texas M. D. Anderson Cancer Center Houston, Texas 77030			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Commander U.S. Army Medical Research and Materiel Command Fort Detrick, Frederick, Maryland 21702-5012			10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200) Breast cancer represents a major cause of death for women in the United States. Overexpression of HER-2/neu oncogene was found in approximately 30% of breast tumor tissues and shown to be a marker indicating poor prognosis for breast cancer patients. HER-2/neu overexpression in cancer cells is also known to enhance cancer metastasis and to induce chemoresistance to certain anti-cancer drugs and repression of HER-2/neu expression reduces malignancy of the cancer cells. Therefore, HER-2/neu overexpression serves as an excellent target for development of breast cancer therapy. Ribozymes have been successfully used to control gene expression. We have designed a novel suicide ribozyme that will allow a gene of interest (such as a toxin gene) to be expressed specifically in the HER-2/neu-overexpressing breast cancer cells, and therefore, will kill only the HER-2/neu-overexpressing cells. This final report describes the progress in the following specific aims: 1) Design of the suicide ribozyme and proof of concept <i>in vitro</i> ; 2) Proof of concept <i>in vivo</i> : a reporter gene regulated by the suicide ribozyme will be expressed only in cells overexpressing HER-2/neu mRNA. 3) Application of concept <i>in vivo</i> : a toxin gene regulated by the suicide ribozyme will preferentially inhibit the growth of breast cancer cells that overexpress HER-2/neu. During the last funding period (9/1/97 - 8/31/98), we have tested our suicide ribozymes in breast cancer cell lines that express either high or low level of HER-2/neu mRNA. Although we have not obtained the optimal suicide ribozyme that would consistently show a preferential expression in HER-2/neu overexpressors, our alternative approach using a HER-2/neu antisense iron responsive element has yielded an encouraging result which may lead to a potential gene therapy treatment against HER-2/neu-overexpressing breast cancer cells.				
14. SUBJECT TERMS Breast Cancer			15. NUMBER OF PAGES 50	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

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INTRODUCTION

A. Background

1. Breast cancer is a major cause of death in the United States.

Data from the Surveillance, Epidemiology, and End Results (SEER) Program has indicated that breast cancer remains a major cause of death in this country (1, 2). It was estimated that more than 190,000 new cases of breast cancer would be diagnosed and approximately 50,000 women would die from this disease in the United States this year. It was also predicated that one in eight women will have breast cancer in their life span in the United States. **Therefore, there is an urgent need to develop novel anti-cancer agents to treat breast cancer.**

2. Amplification and overexpression of the HER-2/neu gene are frequently found in human breast cancer and other types of cancer

Amplification/overexpression of the HER-2/neu (also known as c-erb B2) gene was first found in approximately 30% of human breast cancer (3-8). Several reports have revealed that the overall survival rate and time of relapse for those patients with HER-2/neu - overexpressing breast cancer is significantly shorter than those patients whose tumors do not overexpress HER-2/neu, indicating that HER-2/neu overexpression is an excellent prognostic marker (3, 8). **In addition to breast cancer, HER-2/neu overexpression has also been found in ovarian, lung, gastric, bladder, and oral cancers with high frequency (9-15).**

3. HER-2/neu overexpression provides an excellent target for cancer gene therapy

Gene therapy is an emerging field to develop novel strategies for cancer therapy. The P.I.'s laboratory has a long-standing interest in the role of HER-2/neu oncogene in cellular transformation, and has previously shown that a transcriptional repressor of HER-2/neu oncogene can function as a tumor suppressor for HER-2/neu-overexpressing cancer cells by delivering the gene encoding the transcriptional repressor with either adenoviral or

cationic liposome delivery system(16-18). These studies using animal models have revealed promising therapeutic effect (70%-80% of mice live longer than one year compared with control mice of which 100% died within a few months). However, both adenoviral and liposome delivery systems do not specifically target cancer cells, i.e. the tumor suppressor gene could be delivered into normal cells and potentially result in cytotoxicity. One way to overcome this problem is to design a specific delivery system for HER-2/*neu*-overexpressing cancer cells, such as immunoliposome that contains an antibody to target the HER-2/*neu*-encoded p185 surface protein (19). Alternatively, one may design an expression vector that will express a tumor suppressor gene or toxin gene only in the HER-2/*neu*-overexpressing cancer cells but not in normal cells. **We originally proposed to target HER-2/*neu*-overexpressing breast cancer cells by designing a suicide ribozyme that will specifically express a toxin gene in cancer cells. The suicide ribozyme approach works very well *in vitro* (in test tubes), however, it somehow does not work *in vivo* (in tissue culture). Therefore, we have developed another approach using a HER-2/*neu* antisense iron responsive element which seems to work well *in vivo*. A manuscript describing this novel approach has been published in *Biochem. Biophys. Res. Comm.* this year. The reprint is attached in lieu of a detailed description of this method. (Please also see the Body Section #4)**

4. Ribozyme can be used to control gene expression.

Ribozyme was originally discovered in small RNA pathogens (*Tetrahymena thermophila*) of plants which can form a secondary structure resembling hammerhead and then self-cleave at the 3' side to the GUX triplet (where X can be C, A, or U) (20, 21). This secondary structure contains double-stranded RNA flanking sequences in which a sequence containing 5'GUX3' triplet should be located in one strand to serve as a substrate for cleavage (please see Fig.1 for a ribozyme structure). Artificially synthesized hammerhead

RNA have been proven to behave like the natural ribozyme and can reduce the expression of the target RNA by cleavage of the target RNA sequence (22-24). Hence, ribozyme has become a useful technique to control gene expression by cleavage of target RNAs. However, direct application of the ribozyme (or antisense) approach to target an overexpressed RNA in cells is technically difficult as large number of ribozyme (or antisense oligonucleotides or antisense RNA) molecules will be needed to block the expression of the overexpressed RNA. For example, in many HER-2/*neu*-overexpressing breast cancer cells, the overexpressed HER-2/*neu* mRNA is 100 fold more than that of normal breast epithelial cells (25). It will be much more difficult to directly block expression of HER-2/*neu* oncogene than that of other oncogenes which are expressed at lower levels in cancer cells using the ribozyme or antisense approaches. **We have proposed to take the advantage of the fact that an excess amount of HER-2/*neu* mRNA exists in the breast cancer cells and design a suicide ribozyme that will express a gene of interest only in the presence of overexpressed HER-2/*neu* mRNA. When this suicide ribozyme is fused to a gene encoding a toxin protein (or tumor suppressor), the toxin protein (or tumor suppressor) is predicted to be expressed only in the HER-2/*neu*-overexpressing breast cancer cells and kill the cancer cells. As mentioned earlier, the approach using iron responsive element seems to work better than the ribozyme approach under our experimental conditions.**

B. The purposes

The original proposal will develop a suicide ribozyme targeting at HER-2/*neu*-overexpressing breast cancer cells. The original purposes are:

1. To design a suicide ribozyme structure and demonstrate the predicted ribozyme activity *in vitro*.

2. Proof of concept *in vivo*: to show that a reporter gene regulated by the suicide ribozyme is expressed only in breast cancer cell lines overexpressing HER-2/*neu* mRNA.
3. Application of concept *in vivo*: a toxin gene regulated by the suicide ribozyme will preferentially kill the breast cancer cells that overexpress HER-2/*neu* mRNA.

We also recently developed another approach using the iron responsive element that also provides an expression vector preferentially expressing a gene of interest in HER-2/*neu*-overexpressing breast cancer cells. The result will also be described later.

BODY

1. To design a suicide ribozyme structure and demonstrate the predicted ribozyme activity *in vitro*.

1.a. Design and working principle of the suicide ribozyme

The self-cleaving ribozyme, which is schematically shown in Fig. 1, contains 3 major portions : the suicide ribozyme, a therapeutic gene of interest, and a polyadenylation signal from 5' to 3' direction . The suicide ribozyme is consist of five domains: I, the 5' flanking binding sequence; II, the hammerhead catalytic domain sequence; III, the 3' flanking binding sequence; VI, the loop sequence and V, the substrate sequence from 5' to 3' direction. The two flanking binding sequences (I and III) are derived from the complementary strand of the human HER-2/*neu* sequence so that they can form base pairs with the substrate sequence (part V) derived from human HER-2/*neu* mRNA. The catalytic domain (II) is chosen from the hammerhead ribozyme stem II which will catalyze the cleavage when the secondary structure showed in Fig. 1.A. is formed. The loop sequences (VI) are 5'AAAAAA 3' sequences which has been previously shown to facilitate the formation of ribozyme secondary structure (26, 27). The substrate sequence (V) is derived from the human HER-2/*neu* sequences which contains 5' GUX 3' in the middle serving as the cleavage site and other sequences forming base pairs with flanking binding sequences (I and III). Using RNA fold program to search for HER-2/*neu* gene sequences that fulfill the requirement for the cleavage sequence V, we have identified 40 regions that may be used as the substrate sequences. The following encouraging results shown in Fig. 2 and 3 were obtained using 5' CCCAGUGUGUCAACUGCAGCC 3' [corresponding to nucleotide 1750 to 1771 of human HER-2/*neu* (5)] as the substrate sequence V and its complementary portion as flanking binding sequence I and III. In the absence of exogenous HER-2/*neu* mRNA, the suicide ribozyme will form the secondary structure as

shown in Fig. 1. A, and the cleavage will occur. As a consequence, the polyadenylation signal that is critical for mRNA stability will no longer be covalently linked to the mRNA of the fused gene containing both therapeutic gene and the suicide ribozyme sequence and the mRNA will become unstable and be quickly degraded. Thus, the protein product of the therapeutic gene cannot be expressed. On the other hand, when a large amount of cellular HER-2/*neu* mRNA exists, it will compete with the substrate sequence (V) to hybridize with the flanking binding sequence (I and III) and a structure depicted in Fig. 1.B. may form. This structure will cleave the cellular HER-2/*neu* mRNA and preserve the polyadenylation sequence. Thus the therapeutic protein can be expressed in an environment containing excess of HER-2/*neu* mRNA.

1.b. Proof of concept using *in vitro* transcribed RNA.

To examine whether the ribozyme designed in Fig. 1 can self-cleave and the cellular HER-2/*neu* RNA can prevent self-cleavage as proposed, we placed the suicide ribozyme sequence (I to V in Fig. 1. A.) in an *in vitro* transcription vector driven by T7 bacterial promoter. When the transcript is synthesized by *in vitro* transcription system (Stratagene, La Jolla, CA), we expect to detect two products with 14 nt and 90 nt in length if self-cleavage occurs. As shown in Fig. 2, the full length transcript (104 nt) was readily detected by 2 minutes after *in vitro* transcription was started (At zero minute no transcript was detected, data not shown). However, a small amount of cleaved product, P1(90 nt) was also detected, indicating that self-cleavage occurred within a very short time after full length transcript was made. While the reaction proceeded, the amount of transcripts were increased as evident from the enhanced intensities from both full length transcript and cleaved products. However, the ratio between the full length transcript and cleaved product (e.g. P1) was decreased. By 16 minutes, approximately 70% of the transcripts were cleaved products (please compare the relative intensity between full length transcript and P1). The P2 product contains only 14 nt and therefore its intensity (which is proportional

to the length of transcript) was too weak to be quantitated. The results demonstrated that the designed ribozyme is associated with high efficiency of self-cleavage. To test whether the self-cleavage activity can be inhibited by HER-2/*neu* RNA, exogenous HER-2/*neu* RNA was added to the *in vitro* transcription system. As shown in Fig. 3, in the presence of exogenous HER-2/*neu* RNA the intensity of the full length transcript was significantly increased (Fig. 3, lanes 2 and 4 compared with lanes 1 and 3), suggesting inhibition of the self-cleavage activity. A better separation between the full length transcript and P1 product in Fig. 3 than in Fig. 2 is due to the difference in the density of polyacrylamide gel (20% Vs 10%, Please see figure legend). These results provide strong evidence that the proposed concept works *in vitro*.

2. Proof of concept *in vivo*.

Based on the encouraging data shown in Figs. 2-3, we further constructed an expression vector using the pcDNA1 vector (Invitrogene, San Diego, CA) that contains CMV promoter and polyadenylation signal from SV40 virus. We placed the ribozyme sequences 3' to the reporter gene encoding chloramphenicol acetyltransferase (CAT) expecting that self-cleavage of the ribozyme would make the CAT-containing mRNA unstable and quickly degraded, therefore, no or very weak CAT activity can be detected. However, in the presence of HER-2/*neu* mRNA, the HER-2/*neu* mRNA will be complementary to the flanking binding sequences and prevent formation of self-cleavage ribozyme. Thus, the CAT-encoding mRNA will be able to produce CAT protein that can be easily detected by enzymatic activity of chloramphenicol acetyltransferase. We have compared the CAT expression efficacy between two human breast cancer cell lines, the HER-2/*neu*-overexpressing MDA-MB-453 cells and the HER-2/*neu* low-expressor, MDA-MB-231 by transfecting the CAT-suicide ribozyme constructs into these cells. The transfection efficiency was normalized by β -gal activity that indicated similar transfection efficiency between these two cell lines (RSV-LacZ plasmid was co-transfected with the CAT-suicide

ribozyme construct to provide β -gal activity for normalization of transfection efficiency). As shown in Fig. 4, we have observed higher CAT activity in MDA-MB-453 cells than in MDA-MB-231 cells. However, when we carried out similar experiments in other breast cancer cell lines such as the HER-2/*neu*-overexpressing MDA-MB-361, BT-474 and SkBr3 cells, and the HER-2/*neu* low-expressing MDA-MB-435, MCF-7 cells, we did not observe higher CAT activity in the HER-2/*neu* overexpressors than in the low-expressors as we originally expected. Since these breast cancer cell lines were established from different breast cancer patients, they may have other genetic differences in addition to the different HER-2/*neu* expression. One possible explanation for this inconsistency may be due to an improper location of the suicide ribozyme at the 3' end of the reporter gene since a possible trans-polyadenylation may occur even after the ribozyme was self-cleaved in the absence of overexpressed HER-2/*neu* mRNA. While we plan to place the suicide ribozyme at the 5' untranslated region of a reporter gene with various length of antisense sequences to circumvent the problem mentioned above, we have also searched for other approaches. One of the successful methods is to use the iron-response element to make an iron-responsive vector, as mentioned earlier.

3. Application of concept *in vivo*.

Once the optimal suicide ribozyme (or iron-responding vector) is obtained, we will test whether a HER-2/*neu* overexpression-dependent expression vector can be applied to kill the HER-2/*neu*-overexpressing breast cancer cells. The CAT gene will be replaced by a toxin gene, a gene encoding diphtheria toxin A-chain (DT-A) toxin. Expression of the toxin protein in cells will kill the cells by inhibiting protein synthesis (28). Cell killing can be measured by simply counting the number of survival cells. We already obtained the DT-A expression vector that contains both DT-A gene and a selection marker, neomycin-resistant gene. This toxin-ribozyme construct will be transfected into HER-2/*neu* overexpressors and low-expressors, respectively. Since it contains the neomycin-resistant

gene which will enable the transfected cells to be resistant to the antibiotics G418. Transfectants will be selected by G418 and the G418-resistant colonies will be counted. In the HER-2/*neu* -overexpressing breast cancer cells, excess of HER-2/*neu* mRNA will prevent the suicide ribozyme from self-cleaving and therefore allow expression of the toxin gene, which will eventually kill the cells. We expect the number of G418-resistant colonies to be dramatically reduced compared with the control experiments in which the vector containing only the neomycin-resistant gene but not the toxin gene is transfected into the cells. In the HER-2/*neu* low-expressors, the toxin gene can not be expressed (or expressed at a much lower level) because the self-cleavage of the suicide ribozyme (or the nature of iron-responding element) will destabilize the toxin mRNA. Therefore, the number of G418-resistant colonies will be similar to (or slightly less than) that produced by transfection with the neomycin-resistant gene alone. The proposal is based on a simple but novel concept to express a therapeutic protein. One critical point to apply this concept successfully is the balance between self-cleavage and the inhibition of self-cleavage. Low efficiency of self-cleavage will generate leaky activity and insufficient inhibition of self-cleavage may not produce enough stable mRNA to express the therapeutic protein. **Although we have not found an appropriate condition to use the suicide ribozyme *in vivo*, this approach may still work while we find the right way to do it. The two-year funding from the DOD Breast Cancer Research Program certainly provided us with an excellent opportunity to develop both the suicide-ribozyme and iron-responding approaches, which may provide us with a cancer--specific expressing vector for cancer gene therapy.**

4. An alternative approach using iron responsive element to control selective expression in HER2/neu-overexpressing cancer cells

In addition to the ribozyme approach described above, we have successfully developed an approach for targeting HER2-overexpressing cells based on another novel approach which utilizes a translation regulator, the iron responsive element (IRE), to preferentially express the luciferase reporter gene in the HER-2/neu overexpressing breast cancer cell lines. IRE is a stem-loop RNA structure located in the 5' non-coding region of the ferritin mRNA, and was first identified as a negative translation regulator when it binds to a specific cytoplasmic protein (IRP) at low iron level (29). When the intracellular level of iron is raised the IRP is dissociated from the IRE resulting in the activation of ferritin mRNA by becoming associated with polysomes. By base-pairing to a antisense sequence, the IRE stem-loop structure will be abolished and allowing the mRNA to be translated (Figure 5B). A short HER-2/neu coding sequence that is antisense to a consensus sequence of the iron responsive element (IRE) has been identified (Figure 5A).

To test whether this IRE-antisense sequence can abolish the authentic IRE stem loop and facilitate expression of the controlled reporter gene, we have generated a construct that contains a consensus IRE at the 5' untranslated region (5'UTR) of the luciferase gene driven by a cytomegalovirus (CMV) enhancer-promoter (IRE-LUC) (Figure 5C). This particular IRE possesses a twenty-two nucleotides antisense sequence to a region of the human HER-2/neu coding sequence (between +800 and +821) (Figure 5B) (30). As a control, a CMV-driven luciferase gene was also constructed without an IRE (LUC) (Figure 5C). These two constructs along with an internal control CMV-lacZ gene were transfected into cells by electroporation. The luciferase activity was then normalized by the β -galactosidase activity.

The breast cancer cell lines, MDA-MB-435, which expresses a basal level of HER-2/neu mRNA, and MDA-MB-435eb1, which is a stable transfectant of MDA-MB-435 cells with

HER-2/neu gene and expresses a high level of HER-2/neu mRNA, were used to test our hypothesis. As shown in Figure 6A, the luciferase activity of IRE-LUC is 38- and 14-fold less than LUC in MDA-MB-435 and MDA-MB-435 eb1, respectively. This result indicates that the presence of this particular IRE could greatly reduce the luciferase expression as compared with the one without IRE. This observation is consistent with the translation inhibitory function of a regular IRE. While there is no significant difference between these two cell lines in the expression of LUC, an approximately 3-fold higher luciferase activity of IRE-LUC was observed in the HER-2/neu overexpressing MDA-MB-435eb1 cells than in its parental cell line, MDA-MB-435 (Figure 6B). Thus, the preferential expression of IRE-LUC may be contributed by the overexpressed HER-2/neu mRNA, presumably by forming a sense-antisense hybrid with this IRE and abolish its translation inhibitory function. To further demonstrate the utility of this approach to selectively target the HER-2/neu overexpressing cells, we chose several human breast cancer cell lines which are either expressing basal levels of HER-2/neu mRNA, i.e. HBL-100, MDA-MB-435, and MDA-MB-468, or a high level of HER-2/neu, i.e. MDA-MB-453, and AU565, as recipients of the test constructs. The LUC construct showed at least 30-fold higher expression than that of IRE-LUC in these cell lines (data not shown). More importantly, as shown in Figure 7, the relative expression of IRE-LUC is at least 6-fold higher in the high expressors, MDA-MB-453 and AU565, than that in a low expressor, HBL-100; about 4-fold higher than in MDA-MB-435; and about 5-fold higher than in MDA-MB-468.

The design of the antisense IRE is relatively easy since the IRE consensus sequence allows some flexibility in the length of the "lower" stem and in the variability of nucleotide sequence on both "upper" and "lower" stems (Figure 5A). It needs to be tested to see if optimal structures would confer better selectivity of expression than the region reported in this study. For example, although the number of base pairs of the "upper" stem is fixed at five, the "lower" stem, however, could tolerate variable length. By increasing the length of

the "lower" stem, one could presumably achieve better targeting efficiency with higher sequence specificity. It is obvious that once the optimal IRE was obtained *in vitro*, then it could be placed 5' to a therapeutic gene, such as a toxin, and test the construct in animals to determine its efficacy of delivery and the degree of selective killing of tumor cells overexpressing a given gene *in vivo*. Similar to the suicide ribozyme approach described earlier, this approach may provide another way to selectively express a toxin gene in HER2/neu-overexpressing cancer cells. **At the end of the two-year funding, we are happy to find at least one of the approaches (iron-responding element) works *in vivo* (in tissue culture). We will look for other funding to continue this type of study, especially with emphasis on the application to animal models.**

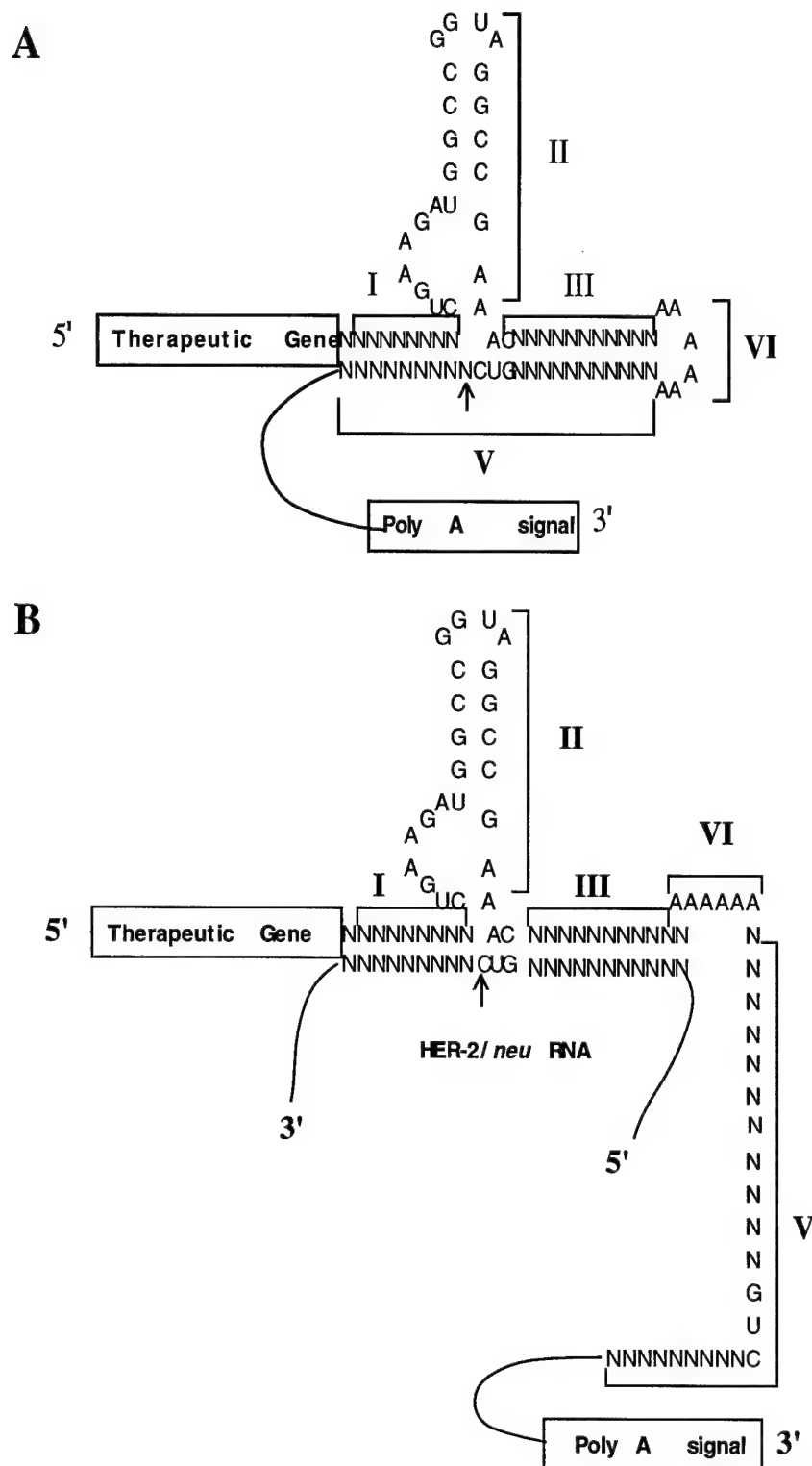


Fig 1. Schematic of suicide ribozyme design. Parts I-V are defined in the text . The arrowheads indicate the cleavage site. Panel A represents a predicted suicide ribozyme structure. Panel B shows a secondary structure between the ribozyme and HER-2/*neu* RNA . This structure will block the self cleavage of suicide ribozyme.

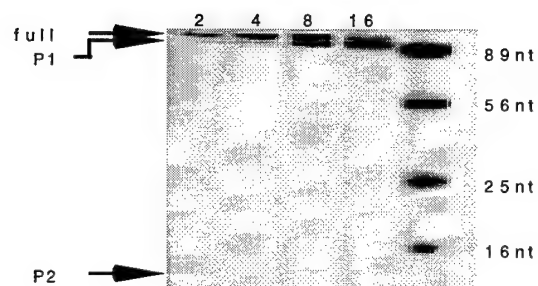


Fig 2. *In vitro* self-cleavage of suicide ribozyme. The suicide ribozyme was *in vitro* transcribed using 32 P-UTP as labeling. At different time point (2, 4, 8, 16 min), the products were stopped by stop buffer and loaded on the 20% 7M urea denatured polyacrymide gel. The self-cleavage was visualized by exposure of the gel to X-ray film. The amount of full length transcripts (Full) were reduced and the cleaved products (P1 and P2) were increased with increasing of the reaction time.

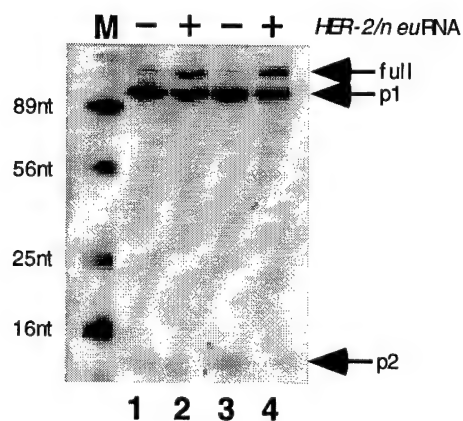


Fig 3. *In vitro* blockage of suicide ribozyme self-cleavage by *HER-2/neu* RNA. The exogenous *HER-2/neu* RNAs were added into the reaction mixture containing 0.5 μ g template DNA encoding ribozyme before *in vitro* transcription. 30 min later, the reaction were stopped by stop buffer. The results were analyzed using 10% 7M urea denatured polyacrymide gel. The materials added to the reaction mixture are: **1.** nonspecific RNA derived from *in vitro* transcription. **2.** *HER-2/neu in vitro* transcripts (*HER-2/neu* : ribozyme 50:1). **3.** 200ng total RNA from the *HER-2/neu* low-expressing cells, MDA-MB-231, or **4.** 200ng total RNA from the *HER/neu*-overexpressing cancer cells, MDA-MB-453. Full: full length suicide ribozyme. P1 and P2: cleavage products. The molecular size standards were indicated on the left.

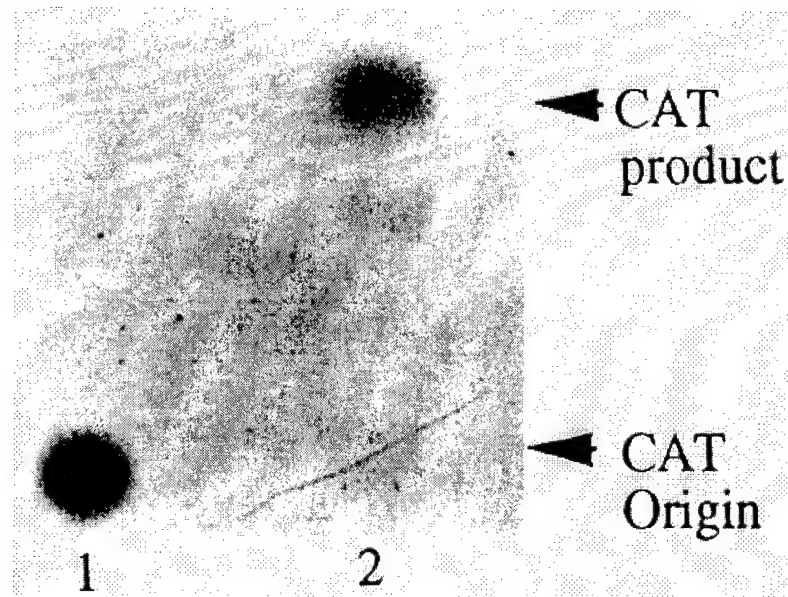


Fig 4. Preferential expression of CAT reporter gene in *HER-2/neu*-overexpressing cancer cells by CAT-suicide ribozyme. 1 μ g CAT-Suicide ribozyme were cotransfected with 4 μ g RSV-LacZ into human breast cancer cell lines with overexpressed *HER-2/neu* (MDA-MB-453) or low-expressed *HER-2/neu* (MDA-MB-231). The total 15 μ g DNA was adjusted by the pGEM3 plasmid. Forty eight hours later, the cells were harvested. The cell extracts normalized by β -gal assay were applied to CAT reaction. Four hours later, the reaction products were loaded on to a TLC plate. Lane 1. *HER-2/neu* low expressor, MDA-MB-231. Lane 2. *HER-2/neu* overexpressor, MDA-MB-453.

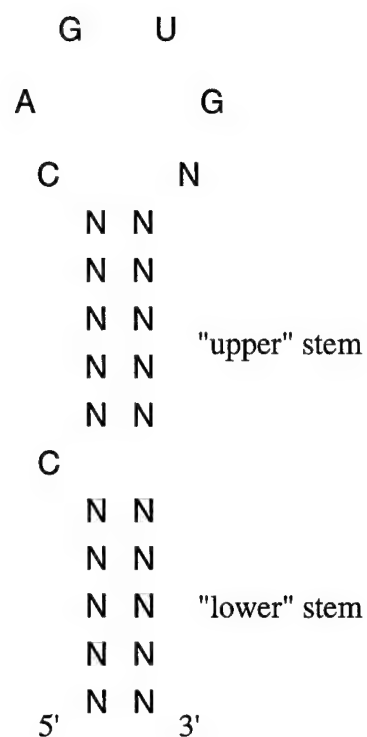


Figure 5A The IRE consensus sequence and stem-loop structure. A six-member loop, the first five bases are usually CAGUG and the sixth base is often a pyrimidine. The "upper" stem usually consists of five base pairs with any complementary nucleotides. A "C" bulge is invariable. The "lower" stem is of various length with any complementary nucleotides but it would also tolerate some unpaired bases.

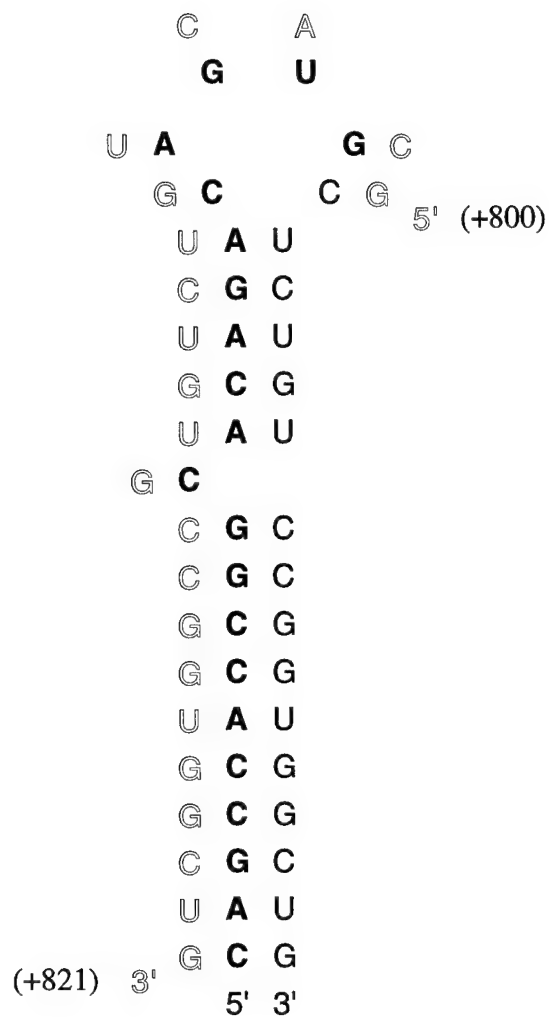


Figure 5B The design of an anti-sense HER-2/neu IRE sequence. The anti-sense sequence to a HER-2/neu coding region is shown in bold face letters. The coding region of HER-2/neu (from +800 to +821) is shown in outlined letters.

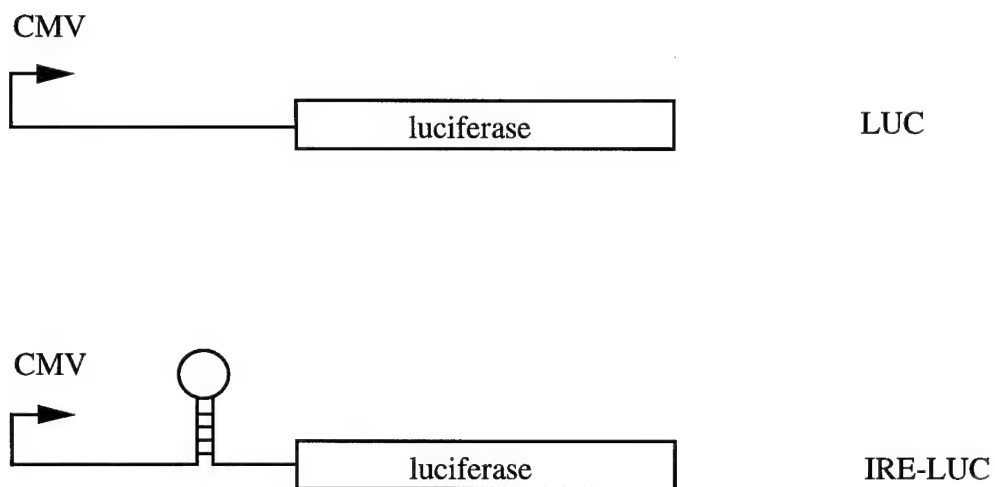


Figure 5C The mRNAs of the luciferase gene driven by CMV promoter either without (LUC) or with (IRE-LUC) IRE located at the 5' UTR.

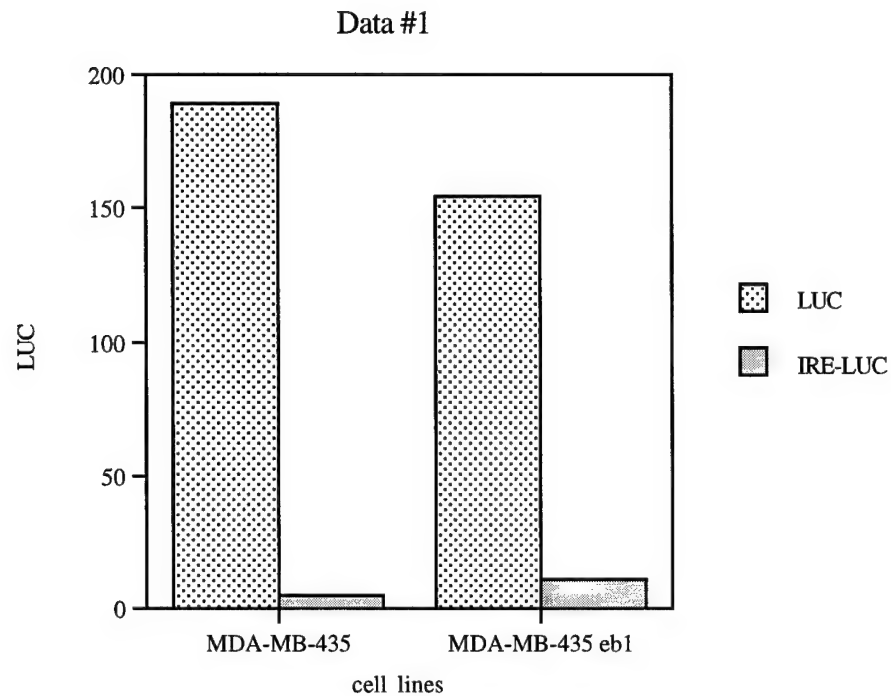


Figure 6A Luciferase activity with or without IRE in MDA-MB-435 and MDA-MB-435eb1. Five micrograms of either LUC or IRE-LUC and two micrograms of CMV-lacZ were electroporated into MDA-MB-435 and MDA-MB-435 eb1 cells. The luciferase activity was assayed 21-24 h after transfection and the adjusted luciferase activity was normalized by the b-galactosidase activity to standardize the transfection efficiency.

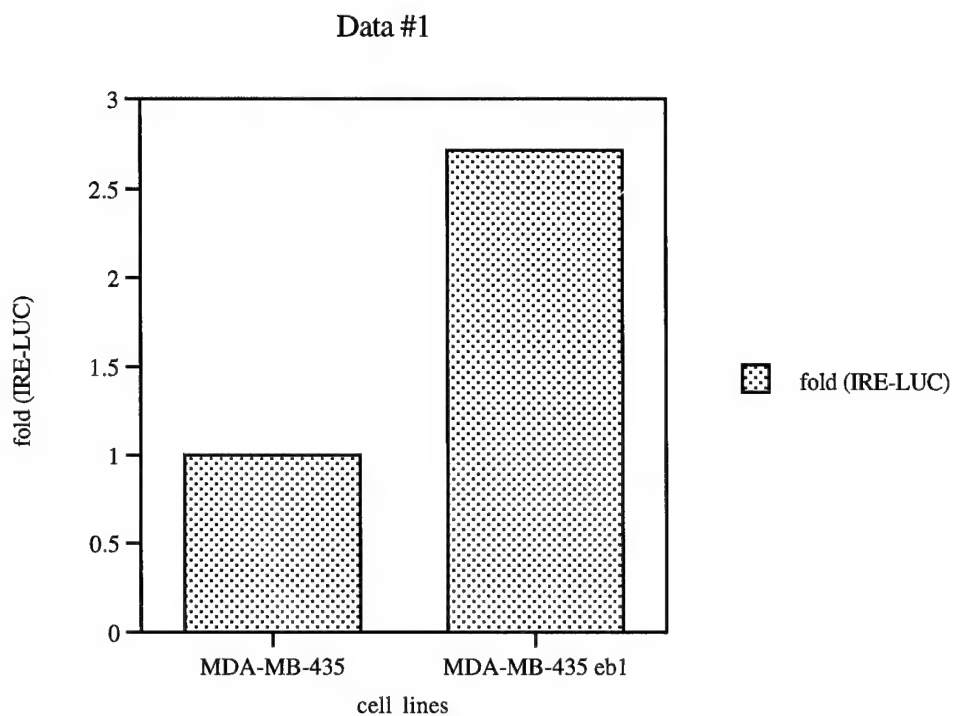


Figure 6B. The fold difference between the luciferase activity of IRE-LUC in MDA-MB-435 and MDA-MB-435 eb1. It was obtained by normalizing both the b-galactosidase activity and the luciferase activity of LUC in both cell lines and set the corrected luciferase activity in MDA-MB-435 as one.

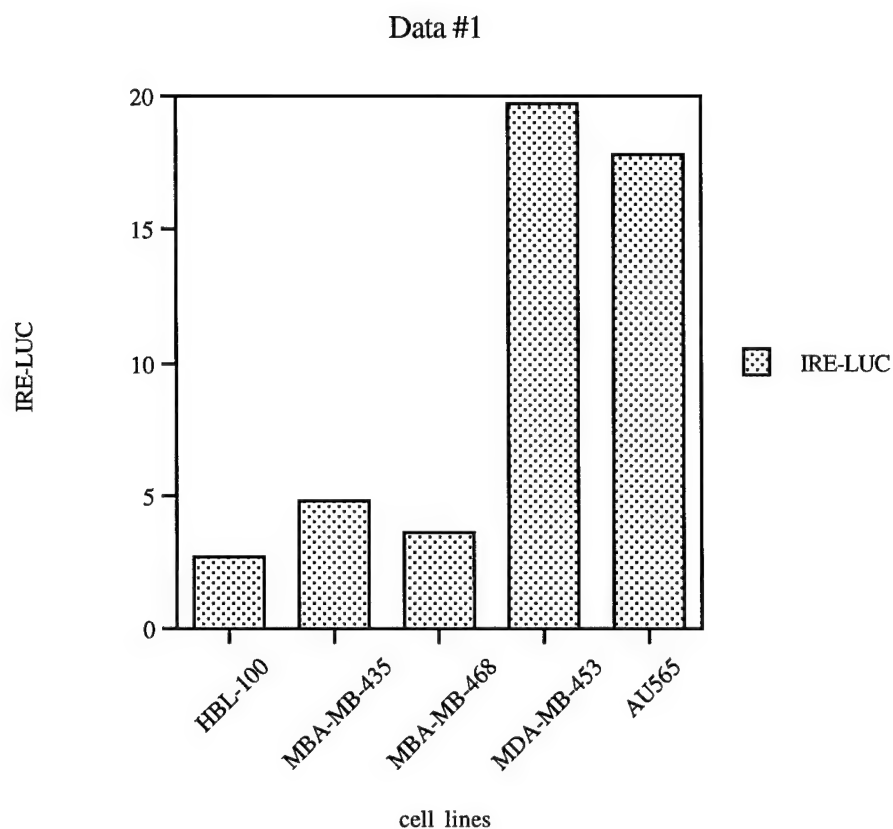


Figure 7. The luciferase activity of IRE-LUC in human breast cancer cell lines. Five micrograms of IRE-LUC and two micrograms of CMV-lacZ were electroporated into several human breast cancer cell lines which either express endogenous level of HER-2/neu mRNA, HBL-100, MDA-MB-435, and MDA-MB-468, or high expressors, MDA-MB-453 and AU565. The luciferase activities have been normalized by the β -galactosidase activities to equalize the transfection efficiency.

CONCLUSION

Task 1: Design of optimal suicide ribozymes

In addition to the suicide ribozyme described in the Specific Aim 1, we have synthesized other constructs that use other regions of the HER-2/neu sequences that also meet the requirement for ribozyme secondary structure to find a optimal suicide ribozyme for the HER-2/neu-overexpressing breast cancer cells. **However, due to the fact that we could not develop an efficient way to express *in vivo*, we have moved our efforts to another approach.**

Task 2: Examination of efficiency of suicide ribozymes *in vitro*

The efficiency of one suicide ribozyme has been tested as shown in Figure 2 and Figure 3.

Task 3: Application of concept *in vivo*

It is not yet clear why the data shown in Figure 4 can only be shown for the MDA-MB-453 and MDA-MB-231 breast cancer cells. And when other cells were used, no preferential expression in the HER-2/neu-overexpressing cancer cells such as MDA-MB-361, BT-474, and SKBr3 can be demonstrated. As described earlier, we have also developed another approach to design a vector for preferential expression in the HER-2/neu-overexpressing cancer cells. As shown in Figures 5-7, this alternative approach using iron response element also provides a way to reach our goal for the development of a vector preferentially expressing a gene in the HER-2/neu-overexpressing breast cancer cells. The concept as well as the preliminary results of this approach has been published recently (please see the attached reprint). We will continue to optimize both approaches to obtain a better vector to reach our goal.

In addition to the one described in the Body section, two other studies relating to HER-2/*neu* oncogene have been completed. The funding support from the current project has been appropriately acknowledged in the resulting publications. These include:

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Final Report for Award Number DAMD17-96-1-6253

LIST OF PERSONNEL:

D. H. Yan

Hua Peng

Targeting Human Breast Cancer Cells That Overexpress HER-2/neu mRNA by an Antisense Iron Responsive Element

Duen-Hwa Yan

*Department of Surgical Oncology and Department of Tumor Biology, The University of Texas
M. D. Anderson Cancer Center, Box 79, 1515 Holcombe Boulevard, Houston, Texas 77030*

Received April 12, 1998

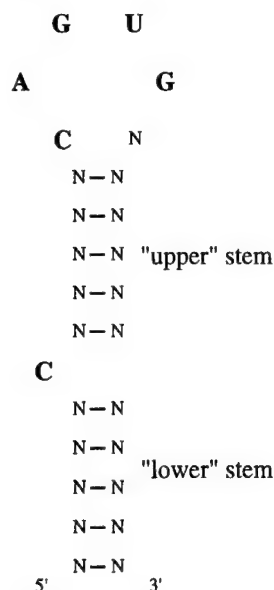
The overexpression of HER-2/neu proto-oncogene has been found in a variety of human cancers. In particular, the amplification and overexpression of HER-2/neu gene were found in 20-30% of breast and ovarian cancer patients with a decreased survival and an increased relapse rates. To target the breast cancer cells overexpressing HER-2/neu mRNA, a novel approach is described that combines the antisense principle and the biochemical property of a translation regulator, an iron responsive element (IRE). This report shows that a HER-2/neu antisense IRE-reporter gene can be preferentially expressed in the breast cancer cells that overexpress HER-2/neu mRNA. This antisense IRE-mediated gene expression system may be applied broadly to target other cell type that uniquely expresses or overexpresses a known gene. © 1998 Academic Press

The human HER-2/neu proto-oncogene is a member of erbB receptor tyrosine kinase family which includes epidermal growth factor receptor (EGFR) (1), erbB3 (2), and erbB4 (3, 4). The HER-2/neu gene encodes a 185 kDa transmembrane tyrosine kinase (p185) which can heterodimerize with erbB3 or erbB4 to form high affinity binding sites for their ligand, heregulin (5, 6). The pathological significance of HER-2/neu expression was realized due to the findings that this gene was overexpressed in human tumors arising from several different sites (7-11). The best example was the observation that about 20-30% of breast and ovarian patients with tumors overexpressing HER-2/neu had a poorer prognosis than those without HER-2/neu overexpression (12-16). These patients had a shorter survival, and had tumors not as responsive to the hormonal and chemotherapy treatments (17-19). Many established human breast cancer cell lines were found possessing an abnormally high level of HER-2/neu expression (20) further suggested an important role of HER-2/neu overexpression in breast tumorigenesis. Therefore, HER-2/neu is a suitable tar-

get for developing effective gene- or immuno-therapy strategy for cancers that overexpress this gene.

Indeed, several methods have been reported to target HER-2/neu overexpressing tumor cells at various step of HER-2/neu biogenesis. For instance, to target p185 receptor protein, either the antibodies against the receptor (21, 22, 41) or heregulin, an erbB family ligand, had been used to efficiently induce cytotoxicity in HER-2/neu overexpressing cells (23, 37, 38). To target p185 tyrosine kinase activity, it has been shown that emodin, a tyrosine kinase inhibitor, not only could suppress transformation and induce differentiation in the HER-2/neu overexpressing cells (31), but also sensitized these cells to chemotherapeutic drugs (44). To target HER-2/neu transcription, several groups had taken advantage of an increased HER-2/neu transcriptional activity in some HER-2/neu overexpressing tumor cells. In one case, estrogen was used to downregulate HER-2/neu expression presumably via transcription repression in breast cancer cells (24). Another group employed a pro-drug strategy that used an expression vector containing cytosine deaminase gene driven by the HER-2/neu promoter. Cytosine deaminase acts to convert a pro-drug, 5-fluorocytosine, to a toxic molecule, 5-fluorouracil. Cells with highly active HER-2/neu promoter activity were killed by 5-fluorocytosine treatment, while cell lines that do not overexpress the gene were unaffected (40). The inhibition of HER-2/neu transcription can also be achieved by using HER-2/neu promoter-specific triplex-forming oligonucleotides that have the ability to interfere with the binding of a sequence-specific transcription activator to the HER-2/neu promoter (43). In addition, using HER-2/neu transcription repressors such as adenovirus E1A (25-29) and SV40 large T antigen (30, 39), our group has demonstrated a significant downregulation of HER-2/neu expression and a reduced tumorigenicity in HER-2/neu overexpressing tumor cells. Finally, to target HER-2/neu mRNA, a ribozyme approach has been recently

A. Consensus IRE



B.

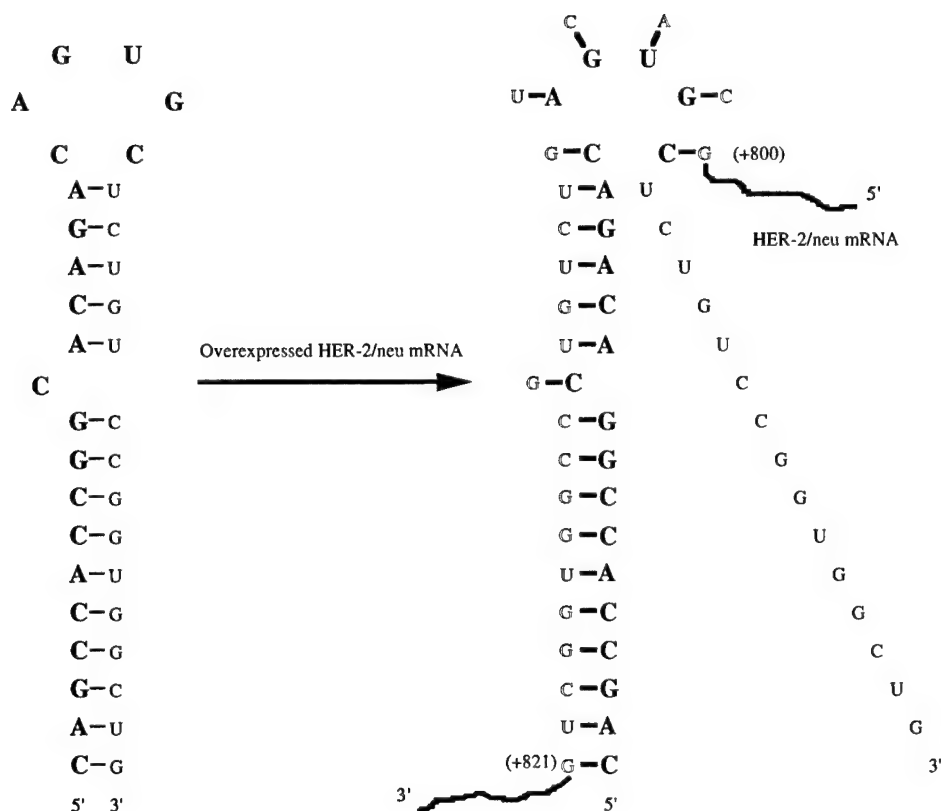
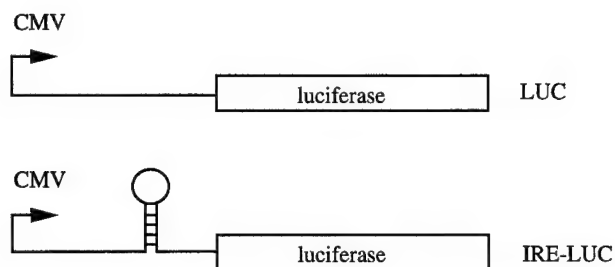


FIG. 1. (A) The IRE consensus sequence and the stem-loop structure (34). A six-member loop, the first five bases are usually CAGUG and the sixth base is often a pyrimidine. The "upper" stem usually consists of five base pairs with any complementary nucleotides. A "C" bulge is invariable. The "lower" stem can be of various length with any complementary nucleotides but it could also tolerate some unpaired bases. (B) A HER-2/neu antisense IRE could be disrupted by the presence of overexpressed HER-2/neu mRNA. The HER-2/neu antisense IRE contains a HER-2/neu antisense sequence (bold face letters) and the complementary nucleotides in the upper and the lower stems (regular letters). A region of the HER-2/neu mRNA (+800 to +821) (outlined letters) can form a sense-antisense hybrid and disrupt the IRE stem-loop structure. (C) The mRNAs of the luciferase gene driven by CMV promoter either without (LUC) or with (IRE-LUC) the HER-2/neu antisense IRE. (D) The regulation of the HER-2/neu antisense IRE by IRF or HER-2/neu mRNA. The binding of IRF to the IRE results in an inhibition of translation of IRE-LUC mRNA. However, this IRF-mediated translation inhibition could be overcome by the presence of overexpressed HER-2/neu mRNA which forms a sense-antisense hybrid with the IRE. The subsequent restoration of translation may be resulted by preventing IRF from binding to the mRNA.

C.



D.

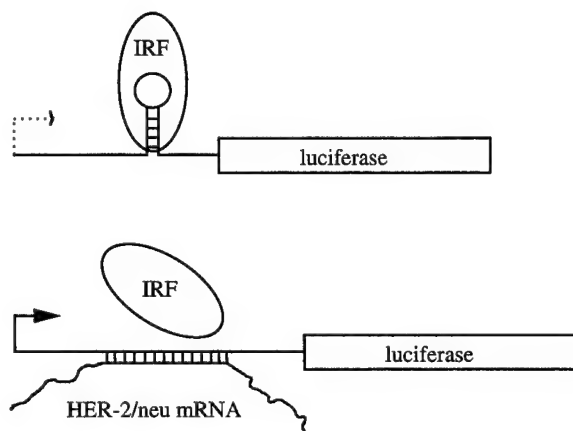


FIG. 1—Continued

reported and showed a dramatic reduction of HER-2/neu mRNA and p185 protein, and a reduced tumorigenicity, in a ribozyme-transfected HER-2/neu-overexpressing ovarian cancer cell line, SKOV-3 (42).

This report describes a novel approach using a HER-2/neu antisense iron response element (IRE) to target the breast cancer cells overexpressing HER-2/neu mRNA. The results showed a preferential expression of an IRE-reporter gene in the HER-2/neu overexpressing breast cancer cells. This method may be used to target other diseases that have a unique expression or overexpression of an identified gene.

MATERIALS AND METHODS

Cell lines. Human breast cancer cell lines, MDA-MB-435, MDA-MB-435 eb1, MDA-MB-468, MDA-MB-453, and AU565, and an immortalized breast cell line, HBL-100, were cultured in DMEM/F12 and 10% fetal bovine serum. The level of HER-2/neu expression in each cell line has been documented previously (19, 20, 31). MDA-MB-435 eb1 cell line was generated by transfecting MDA-MB-435 cells with a human HER-2/neu cDNA driven by CMV promoter (19).

Construction of plasmids. To make a luciferase expression plasmid, LUC, a luciferase gene was obtained by restricting pGEM-luc

plasmid (Promega) with HindIII and XhoI followed by subcloning this gene into the same sites of a pcDNA3 plasmid (Invitrogen). The IRE oligonucleotide (upper strand) used in this study has the following sequence, 5'GGCCGCCAGCCACCGGCACAGACAGTGC-TCTGTCCGGTGGCTGG3'. The bold letters are the IRE consensus nucleotides and the underlined region is an antisense sequence of a HER-2/neu coding region located from +800 to +821 (35, Figure 1B). The IRE oligonucleotides are flanked by NotI (5') and BamHI (3') restriction sites so that IRE-LUC plasmid could be made by subcloning the IRE into the same sites on the 5' UTR of the LUC plasmid. The presence of the IRE in IRE-LUC was confirmed by DNA sequencing. The internal control plasmid, CMV-lacZ, was reported previously (32).

Electroporation. For each transfection, $2 - 5 \times 10^6$ cells were harvested by trypsinization and resuspended in 500 μ l of 1 \times PBS in an ice-cold 2mm gap cuvette (BTX Electroporation System, San Diego, CA). Five micrograms of either LUC or IRE-LUC and two micrograms of CMV-lacZ were added to the cells and electroporated at 350 V and 400 μ F in an Electro Cell Manipulator 600 (BTX Electroporation System). The transfected cells were immediately resuspended in a well of a six-well plate that contains 5 ml of DMEM-F12 medium and 10% fetal calf serum in each well.

Luciferase and β -galactosidase assays. 21-24 hrs after electroporation, the medium was removed and cells were washed once with 1 \times PBS before lysis. Cell lysates were obtained by adding 100 μ l of Reporter Lysis Buffer (Promega) to each well and the crude cell extracts were frozen and thawed once followed by microcentrifugation. The supernatants were used for the subsequent assays. For the luciferase assay, 50 μ l of lysate was mixed with 50 μ l of luciferase substrate (Promega) prior to light detection by a luminometer (TD-20/20, Promega). Ten second measurement of light intensity began at 5 seconds after mixing lysate and substrate together. The β -galactosidase assay, using 20 μ l of lysate, was done as reported previously (33).

RESULTS AND DISCUSSION

IREs are a class of translation regulators that all possess a stem loop structure (Figure 1A) and are all regulated by iron (reviewed in 34 and references therein). The first IRE described in the literature was found to be located at the 5' non-coding region of an iron storage protein, ferritin. It functions as a negative translation regulator when bound to the specific cytoplasmic protein (IRP) in the presence of low cytosolic iron concentration. When the intracellular level of iron is raised, the IRP is dissociated from the IRE resulting in the activation of ferritin mRNA. The freed mRNA then becomes associated with polysomes allowing it to be translated. Ferritin, in turn, protects cells from the toxic effects of iron by sequestering the mineral within its hollow shell.

In an attempt to develop a gene expression system that could be specifically activated in HER-2/neu overexpressing cells, we incorporated the unique translation regulatory properties of an IRE into a HER-2/neu antisense targeting scheme. In contrast to the IRE-mediated translational regulation by iron, this IRE-mediated gene expression system was designed in a way that the gene can also be upregulated by the presence of an excess amount of HER-2/neu mRNA. Namely, by placing a HER-2/neu antisense IRE in the 5' UTR of a gene, the translation inhibition of this gene

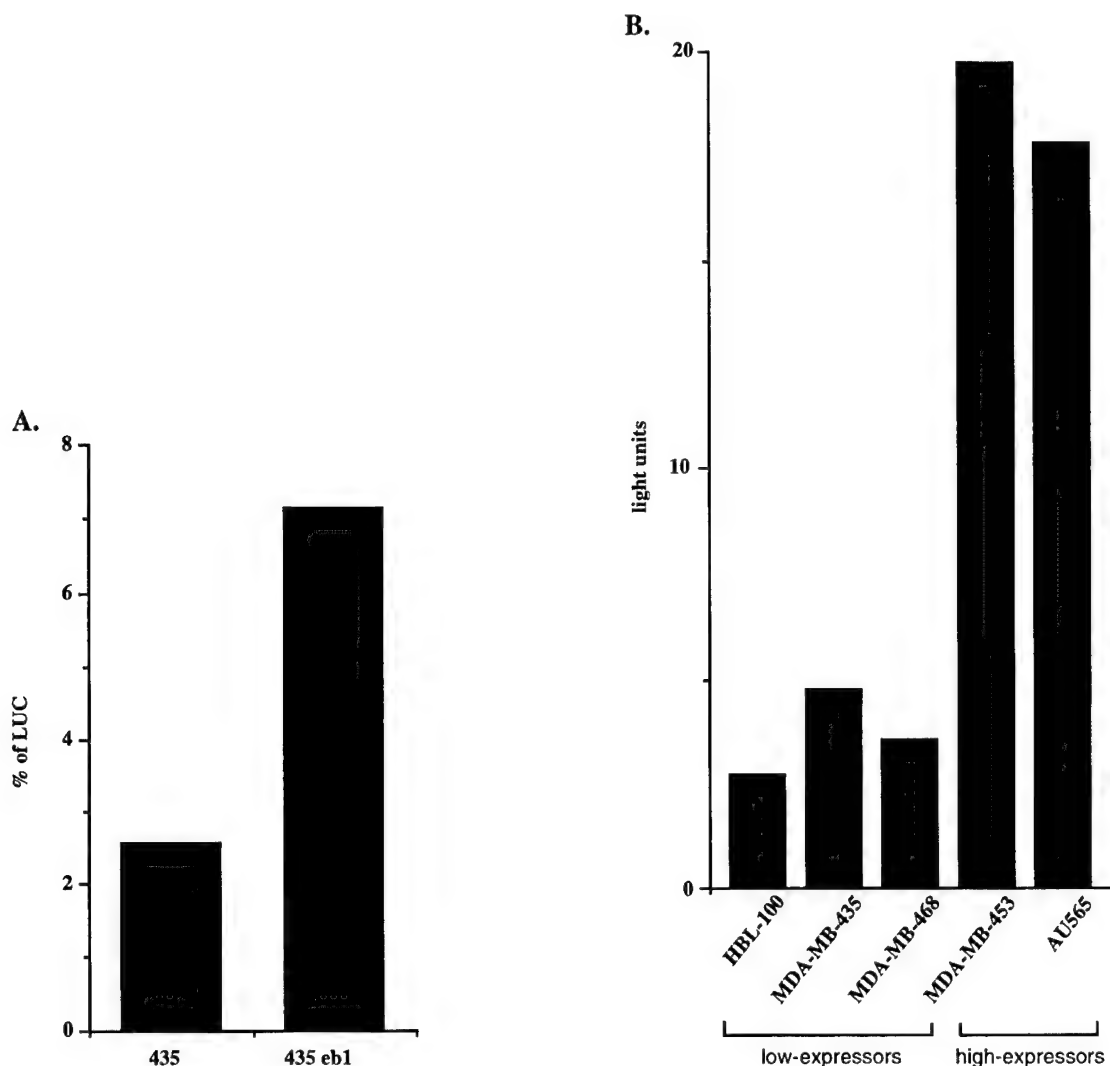


FIG. 2. (A) The luciferase activity of LUC or IRE-LUC in MDA-MB-435 and MDA-MB-435 eb1. Five micrograms of either LUC or IRE-LUC and two micrograms of CMV-lacZ were electroporated into MDA-MB-435 (435) and MDA-MB-435 eb1 (435 eb1) cells. The luciferase activity was measured 21-24 h after transfection and normalized for transfection efficiency by the β -galactosidase activity. The relative luciferase activity of IRE-LUC (% of LUC) was calculated by setting the luciferase activity of LUC obtained from each cell line as 100%. (B) The luciferase activity of IRE-LUC in human breast cancer cell lines. Five micrograms of IRE-LUC and two micrograms of CMV-lacZ were electroporated into human breast cancer cell lines: the low HER-2/neu mRNA expressors, HBL-100, MDA-MB-435, and MDA-MB-468, and the high expressors, MDA-MB-453 and AU565. The luciferase activity was normalized for transfection efficiency by the β -galactosidase activity.

can be overcome in a HER-2/neu mRNA overexpressing cell. Presumably, this may be accomplished by disrupting the IRP-IRE interaction when HER-2/neu mRNA hybridizes to the IRE (Figure 1D). To test this concept, a CMV-driven plasmid, IRE-LUC, containing a HER-2/neu antisense IRE in the 5' UTR of the luciferase gene was constructed (Figure 1C). This recombinant IRE possesses a twenty-two nucleotide antisense sequence to a region of a human HER-2/neu coding sequence between +800 and +821 (Figure 1B, Materials and Methods) (35). A CMV-driven luciferase gene, LUC, was made to serve as a control (Figure 1C). To test the feasibility of this approach in a model system,

IRE-LUC and LUC were transfected into two breast cancer cell lines with identical genetic background: MDA-MB-435 expresses a low level of endogenous HER-2/neu mRNA; and MDA-MB-435 eb1 (36), which is a MDA-MB-435 stable transfectant, expresses a high level of transfected HER-2/neu protein. Figure 2A shows a 3-fold higher luciferase activity of IRE-LUC in MDA-MB-435 eb1 as compared to the parental low expressor, MDA-MB-435. Although the control plasmid, LUC, expressed readily in both cell lines (data not shown), the presence of the IRE greatly reduced the luciferase expression, i.e. 38-fold less and 14-fold less than that of LUC in MDA-MB-435 and MDA-MB-435

eb1 respectively. This observation is consistent with the translation inhibitory function of a classical IRE (34). This data suggests that the presence of overexpressed HER-2/neu mRNA may be responsible for the preferential expression of IRE-LUC in MDA-MB-435 eb1 cells. To explore a general usefulness of this approach, several human breast cancer cell lines with either low or high endogenous level of HER-2/neu mRNA (20) were examined by transfecting IRE-LUC into these cells. The results are shown in Figure 2B. The high expressors, MDA-MB-453 and AU565, show at least 4-fold increase in luciferase activity as compared to the low expressors, HBL-100, MDA-MB-435, and MDA-MB-468. Thus, this observation further supports the idea that IRE-LUC can be expressed preferentially in cells that overexpress HER-2/neu mRNA.

Here, a novel antisense strategy was used to selectively target the breast cancer cells with the overexpression of HER-2/neu mRNA. This approach may be applied to other systems in which a targeted cell overexpresses a known gene. The design of an antisense IRE is relatively easy because the IRE consensus sequence allows certain flexibility in the length of the "lower" stem and, more importantly, permits variability of nucleotide sequence in both "upper" and "lower" stems (Figure 1A). To make this system work, the targeted mRNA should have an antisense sequence that would match the IRE consensus sequence. The odds are reasonable for an average length mRNA that this sequence can be found. Several features of an IRE consensus sequence, ${}^5\text{NNN-NNCNNNNNCAGUGNNNNNNNNNN}{}^{3'}$, need to be considered for designing an antisense IRE: (a) the bold-typed C and CAGUG are nearly invariable. (b) the underlined sequence denotes the nearly always a five base interval between the C and CAGUG. (c) the italicized nucleotides represent the bases that are complementary, or antisensed, to the mRNA of interest. (d) the non-italicized Ns were designed to complement the italicized Ns, except the bold-typed nucleotides, in a way that it would form a stem loop structure (Figure 1A). The design of the antisense IRE illustrates the great flexibility and adaptability of this system.

To our knowledge, this is the first study that shows that an IRE can be engineered so that its translation inhibitory effect can be modulated by a molecule other than iron. However, it is possible that more improvements upon the current system could generate a greater difference in IRE-LUC expression between the high- and the low-expressors. For example, although the presence of overexpressed HER-2/neu mRNA in MDA-MB-435 eb1 may account for a 3-fold increase in the luciferase activity of IRE-LUC, but this activity was 14-fold less than that of the control, LUC (Figure 2A), indicating a limited restoration of the mRNA translation. One way to further increase the translation activity of IRE-LUC in the high-expressor may be to increase the hybridization efficiency between HER-

2/neu mRNA and the antisense IRE. This may be accomplished by, first, screening different antisense IREs to test if other antisense sequences may confer a better hybridization efficiency. In the case of HER-2/neu mRNA, two other antisense regions, i.e. from +434 to +454 and from +2881 to +2900 (35), which match the IRE consensus sequence are eligible for further testing. Secondly, since the "lower" stem can tolerate variable lengths and unpaired bases, it is conceivable that an increased length of the lower stem may offer better targeting efficiency. Once an optimal antisense IRE is obtained *in vitro*, it should be placed 5' to a therapeutic gene, such as one that expresses a toxin, and test the degree of selective killing of the breast cancer cells that overexpress HER-2/neu mRNA.

ACKNOWLEDGMENTS

The author thanks Dr. Mien-Chie Hung for his encouragement and generous support for this project. The author also thanks Drs. Ralph Zinner and Michael Van Dyke for their critical reading of the manuscript. This work was supported by grants CA58880 (NIH) and DAMD17-96-I-6253 (Department of Defense) (to M-CH).

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Tyrosine kinase inhibitors, emodin and its derivative repress *HER-2/neu*-induced cellular transformation and metastasis-associated properties

Lisha Zhang¹, Yiu-Keung Lau¹, Larry Xi¹, Ruey-Long Hong^{1,2}, Darrick SHL Kim³, Chieh-Fu Chen⁴, Gabriel N Hortobagyi⁵, Ching-ger Chang³ and Mien-Chie Hung¹

¹Department of Tumor Biology, Box 79, The University of Texas MD Anderson Cancer Center, Houston, Texas 77030, USA;

²Department of Oncology, National Taiwan University Hospital Taipei, Taiwan; ³Department of Medicinal Chemistry and Pharmacognosy, Purdue University, West Lafayette, Indiana 47907, USA; ⁴National Research Institute of Chinese Medicine and National Yang-Ming Medical College, Shih-Pai Taipei (11211) ROC; ⁵Department of Breast Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas 77030, USA

We have previously shown that emodin suppresses tyrosine kinase activity of *HER-2/neu*-encoded p185^{neu} receptor tyrosine kinase. In this study, we examine the relationship between the chemical structure and the activity of emodin and nine derivatives, and identified that one methyl, one hydroxy, and one carbonyl functional groups are critical for the biological activities of emodin. We also found that one of the derivatives 10-(4-acetamidobenzylidene)-9-anthrone (DK-V-47) is more effective than emodin in repressing the tyrosine phosphorylation of p185^{neu} and in inhibiting the proliferation and transformation of *HER-2/neu*-overexpressing human breast cancer cells. Using mutation-activated *HER-2/neu* transformed 3T3 cells, we also investigated whether emodin and DK-V-47 can inhibit malignant transformation induced solely by the *HER-2/neu* oncogene. We found that DK-V-47 is more potent than emodin in suppressing transformation phenotypes of activated *HER-2/neu* transformed 3T3 cells including anchorage-dependent and -independent growth, metastasis-associated properties. These results clearly indicate that the inhibition of p185^{neu} tyrosine kinase by both emodin and DK-V-47 is capable of suppressing the *HER-2/neu* associated transformed phenotypes including the ability to induce metastatic potential. Our results also support the chemotherapeutic implications of the use of either emodin or DK-V-47 to target *HER-2/neu*-overexpressing cancer cells.

Keywords: emodin; anthraquinone; *HER-2/neu*; tyrosine kinase; experimental metastasis

Introduction

The *HER-2/neu* gene (also known as *c-erbB-2*) encodes a 185^{neu} transmembrane tyrosine kinase (p185^{neu}) homologous to epidermal growth factor receptor (EGFR) (Hung *et al.*, 1986; Coussens *et al.*, 1985; Bargmann *et al.*, 1986; Semba *et al.*, 1985; Yamamoto *et al.*, 1986). Amplification and/or overexpression of *HER-2/neu* has been detected frequently in many human cancers (Slamon *et al.*, 1987, 1989; Zhang *et al.*

et al., 1989; Schneider *et al.*, 1989; Weiner *et al.*, 1990; Yokota *et al.*, 1988; Chau *et al.*, 1990; Hou *et al.*, 1992; Myers *et al.*, 1994). Its enhanced expression is correlated with chemotherapeutic drug resistance in some experimental systems (Yu *et al.*, 1996; Tsai *et al.*, 1993, 1995), number of lymph node metastases in node positive breast cancer patients (Slamon *et al.*, 1987; Lacroix *et al.*, 1989; Tauchi *et al.*, 1989) and shortened survival in breast, ovarian, lung, oral and gastric cancer patients (Sadasivan *et al.*, 1993; Veltri *et al.*, 1994; McCann *et al.*, 1991; Vijver *et al.*, 1987; Gusterson *et al.*, 1992; Toikkanen *et al.*, 1992; Berchuck *et al.*, 1990; Xia *et al.*, 1997; Yoshida *et al.*, 1989).

Cellular and animal experiments have shown that an increase in *HER-2/neu* expression enhances malignant phenotypes of cancer cells, including metastatic potential (Yu and Hung, 1991; Yu and Hung, 1991; Yu *et al.*, 1994; Yusa *et al.*, 1990). The association of *HER-2/neu* overexpression in cancer cells with chemoresistance and metastasis provides a plausible interpretation for the poor clinical outcome of patients with *HER-2/neu*-overexpressing cancers; it suggests that enhanced tyrosine kinase activity of *HER-2/neu* plays a critical role in the initiation, progression, and outcome of human tumors.

Emodin (3 methyl-1,6,8 trihydroxyanthraquinone) is an inhibitor of protein tyrosine kinase (Jayasuriya *et al.*, 1992) which was isolated from *Polygonum Cuspidatum*. Emodin was recently shown to suppress *HER-2/neu* tyrosine kinase activity in *HER-2/neu*-overexpressing human, breast and lung cancer cells (Zhang *et al.*, 1995; Zhang and Hung, 1996). It has also been shown to sensitize *HER-2/neu*-overexpressing lung cancer cells to chemotherapeutic drugs (Zhang and Hung, 1996).

In an attempt to search for compounds more effective than emodin to inhibit *HER-2/neu* tyrosine kinase activity and cell transformation, we examined the relationship between the chemical structure and activity of the derivatives of emodin on tyrosine phosphorylation of *HER-2/neu*. We found that one of nine of the derivatives, 10-(4-acetamidobenzylidene)-9-anthrone (DK-V-47) is more effective than emodin in repressing tyrosine phosphorylation of p185^{neu}, in inhibiting growth, and in suppressing the transformation phenotypes of these cancer cells. Because it is not clear whether emodin and DK-V-47 might also exhibit other biological activity which could also contribute to the suppression of the malignance of human cancer

cells, it is critical to determine whether the two compounds can inhibit malignant transformation induced solely by the *HER-2/neu* oncogene. To address this issue, we included *HER-2/neu* transformed 3T3 cells in our study.

Results

Effect of emodin and its derivatives on *HER-2/neu*-overexpressing human breast cancer MDA-MB 453 cells

To determine the relationship between chemical structures of emodin and its derivatives and their inhibitory activities on the tyrosine phosphorylation of *HER-2/neu* and on the proliferation of *HER-2/neu*-overexpressing breast cancer cells, we synthesized nine derivatives as shown in Figure 1. We separated the derivatives of emodin into four groups, according to substitute at the different structural position of emodin. We treated human breast cancer MDA-MB 453 cells that overexpress p185^{neu} with varying concentrations of ten compounds at 37°C for 24 h in the absence of serum. We then analysed the ten compounds to determine the protein level of p185^{neu} and its tyrosine phosphorylation, we used immunoblotting with anti-phosphotyrosine antibody for the detection of phosphotyrosine (anti-PY) and anti-p185^{neu} antibody for the detection of p185^{neu}. As summarized in the Table 1, of the ten compounds tested the carbon 10 (C₁₀) group substitute DK-V-47 was revealed to be the most effective in suppressing the tyrosine phosphorylation of p185^{neu}, the parent compound emodin is slightly less effective. To achieve a 50% inhibitory activity of tyrosine phosphorylation, 21 µM of emodin and 17 µM of DK-V-47 are required. Emodin and its nine derivatives under the same condition did not affect the protein levels of p185^{neu} (data not shown).

We also investigated the effects of emodin and its derivatives on the proliferation of MDA-MB 453 cells. Cells were treated with different concentrations of the compounds at 37°C for 72 h and then measured by the MTT assay. As also shown in Table 1, DK-V-47 is the most effective compound inhibiting cell growth; the IC₅₀ of emodin is fivefold higher than that of DK-V-47.

The findings from four groups also indicate that the methyl group at C₃, the hydroxy group at C₆, and the carbonyl group at C₁₀ position are critical for maintaining the activity of emodin required to suppress the tyrosine phosphorylation of *HER-2/neu* and to block the growth of *HER-2/neu*-overexpressing breast cancer cells. However, when the ketone at C₁₀ is replaced by the *p*-acetamidobenzylidene group, the compound DK-V-47 demonstrates more potent activity than does emodin. The CH₃CO group in the backbone of DK-V-47 appear to be critical for the biological activity; replacement of this group by H almost completely abolishes the biological activities.

We have previously shown that, among the different biological assays, suppression by emodin is much more profound in the soft agarose colonization assay; this assay which is used to measure the ability of cells to grow in an anchorage-independent environment, represents transformation status of cells *in vitro*. As shown in Figure 2, when DK-V-47 was examined for its effect on breast cancer cells in a soft agarose

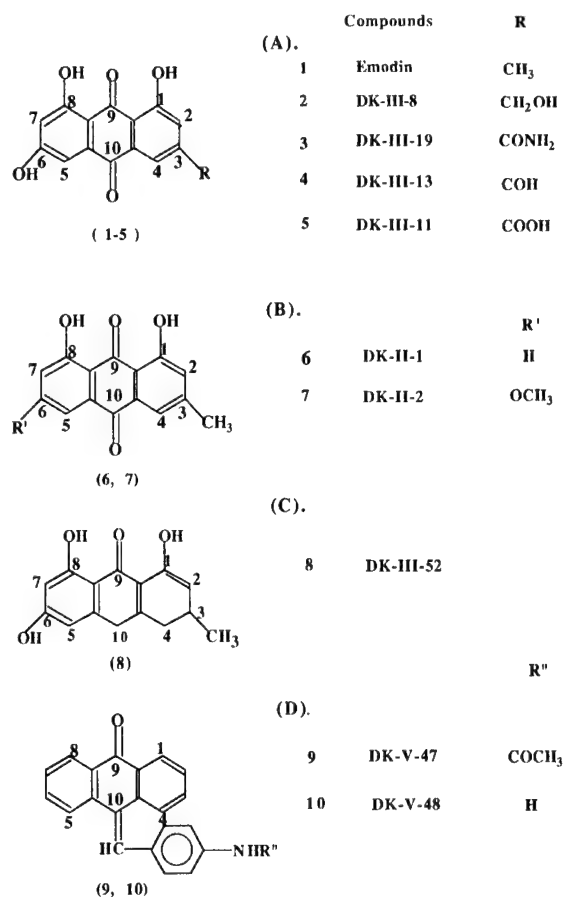


Figure 1 The chemical structures of emodin and its derivatives

Table 1 Relationship between chemical structures of emodin and its derivatives and their inhibitory activities on tyrosine phosphorylation of *HER-2/neu* and proliferation in MDA-MB-453 cells

	Inhibitory activity (IC ₅₀ , in µM) ^a	
	phosphorylation of <i>HER-2/neu</i> ^b	Proliferation of cells ^c
Group A		
Emodin	21	5 + 0.4
DK-III-8	> 100	81 + 6.2
DK-III-19	> 100	45 + 3.6
DK-III-13	100	60 + 5.4
DK-III-11	> 100	> 100
Group B		
DK-II-1	> 100	> 100
DK-II-2	> 100	100 + 9.6
Group C		
DK-III-52	> 100	> 100
Group D		
DK-V-47	17	1 + 0.1
DK-V-48	> 100	> 100

^aConcentration of reagents needed for 50% growth inhibition of cells. ^bCells were treated by different concentrations of emodin and derivatives in the absence of serum for 24 h, then tyrosine phosphorylation of *HER-2/neu* were analysed by Western blot and quantitated by 'NIH Image' software program as described in Materials and methods. ^cCells were treated by different concentrations of emodin and derivatives for 72 h in the presence of serum, then proliferation of cells were measured by MTT assay as described in Materials and methods

colonization assay, the colony formation activity of both breast cancer cells MDA-MB-453 and MCF-7 were suppressed in a dose-dependent manner. However, at the same concentrations of the drug (either at 0.5 or at 2.0 μM) the *HER-2/neu* overexpressing MDA-MB-453 cells (Figure 2a) are much more sensitive to the drugs than the MCF-7 cells (Figure 2b) which express basal levels of p185^{neu} (at 0.5 μM , $P < 0.001$; at 2.0 μM , $P < 0.001$).

In addition, as shown in Figure 2, the activity of DK-V-47 to suppress anchorage-independent growth is much more potent than that of emodin ($P < 0.0001$), for example, at a concentration of 0.5 μM , DK-V-47 shows significant inhibitory activity for the *HER-2/neu*-overexpressing MDA-MB-453 cells in colony formation, to achieve similar inhibition, higher than 20 μM emodin is required. The profound effect of the differential suppression of DK-V-47 and emodin on

MDA-MB-453 cells in the soft agarose colonization assay compared with other biological assays such as the tyrosine phosphorylation of p185^{neu} and the cell proliferation by MTT assay, as shown in Table 1, could be due to the fact that the different incubation times of the compounds with cells are required for different assays; 2–3 weeks are needed for the soft agarose colonization assay, whereas only 2–3 days are needed for other assays.

Emodin and DK-V-47 induce G_0/G_1 arrest

Since cell cycle distribution is closely related to cell proliferation rate, both effective compounds, emodin and DK-V-47 were further subjected to cell cycle distribution analysis (Figure 3). The number of cells in S-phase is apparently reduced and those in G_0/G_1 phase significantly increased. The results indicated that both compounds are able to inhibit DNA replication and to induce cell cycle arrest at G_0/G_1 phase, suggesting that cell cycle arrest may contribute to the growth inhibition.

Cell growth inhibition by emodin is reversible

In order to determine if inhibition of emodin on cell growth is stable or transitory, we treated MDA-MB-453 cells with 40 μM emodin for 3 days in the presence of serum, and washed off the reagents. These cells were then incubated with fresh serum-containing medium along with or without emodin for different time intervals. The viable cells were then measured by MTT assay and the fraction of viable cells was calculated by defining the absorption of cells without treatment of emodin as 100%. As shown in Figure 4, after cells were treated with 40 μM of emodin for 3 days, the fraction of viable cells decreased to 38% compared with untreated cells. For the cells continuously treated with emodin for the additional 5 days, the cell growth remained inhibited. However, when emodin was washed out after 3-day treatment, the cells started to grow and the growth rate was similar to the untreated cells in the subsequent time course. These results indicate that inhibitory effect by emodin is reversible.

Repression of autophosphorylation and transphosphorylation by emodin and DK-V-47 in vitro

While the results just described suggest that tyrosine kinase inhibitors such as emodin and DK-V-47 preferentially suppress the transformation phenotype of *HER-2/neu*-overexpressing MDA-MB-453 human cancer cells, it should be noted that these human cancer cells are derived from different patients and are likely associated with multiple genetic alterations. *HER-2/neu* expression may not be the only genetic difference between these two cancer cell lines.

To specifically investigate the effects of the tyrosine kinase inhibitors on *HER-2/neu* mediated transformation phenotypes, we would need transformed cells of which the transformation phenotypes are induced solely by *HER-2/neu* oncogene. To achieve this, we used an NIH3T3 transfectant, B104-1-1, which was transformed by the transfection of the mutation-activated rat *HER-2/neu* oncogene (Hung et al., 1989;

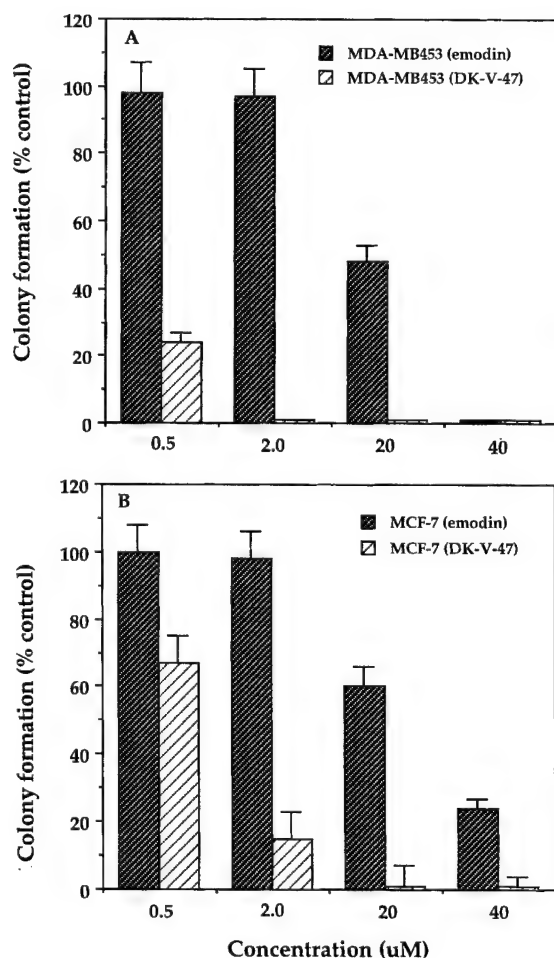


Figure 2 Emodin and DK-V-47 repress colony formation of human breast cancer cells in soft agarose. MDA-MB-453 cells (a) and MCF-7 cells (b) were seeded into 24-well plates (1×10^3 cells/well), in culture medium containing 0.35% agarose over a 0.7% agarose layer with or without different concentrations of emodin or DK-V-47 incubated for 3 weeks at 37°C. Colonies were stained with *p*-iodonitro-tetrazolium violet and counted. The percentage of colony formation was calculated by defining the number of colonies in the absence of either emodin or DK-V-47 as 100%. All measurements were made four times; the results are means \pm s.d.

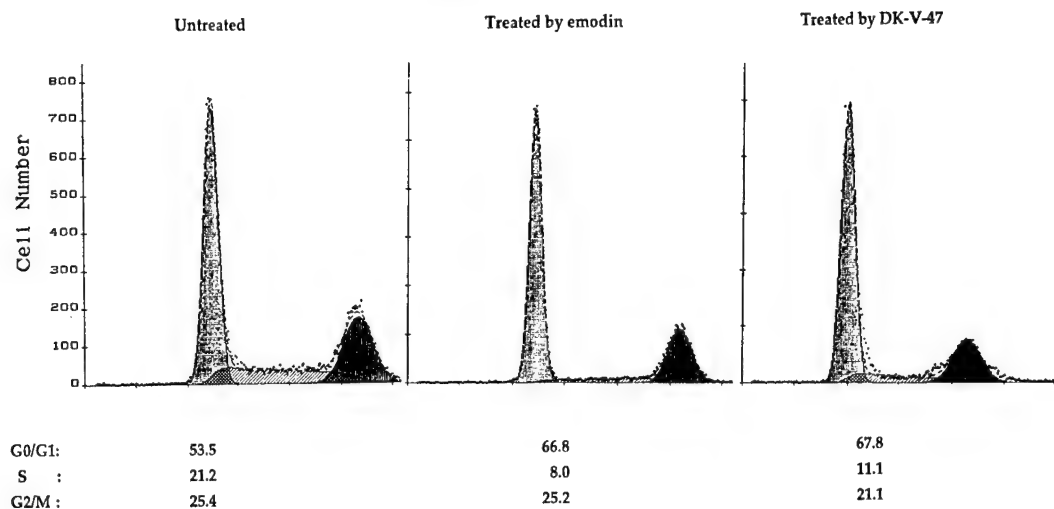


Figure 3 Emodin and DK-V-47 induce G₀/G₁ arrest. MDA-MB 453 cells were treated with or without either emodin (40 μ M) or DK-V-47 (20 μ M) for 3 days and trypsinized, the DNA content analyses were analysed as described under Materials and methods

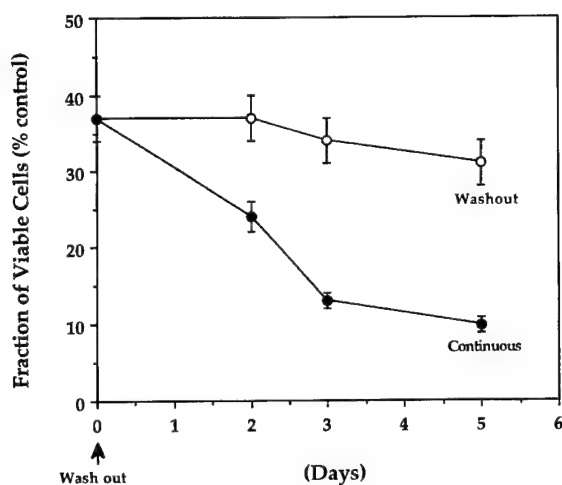


Figure 4 Cell growth inhibition by emodin is reversible. MDA-MB 453 cells were treated with or without 40 μ M emodin in the presence of serum for 3 days, and then washed off the reagents. After that, the cells were incubated with fresh medium either in the presence or absence of emodin for different times, the viable cells were measured by MTT assay, and fraction of viable cells was calculated by defining the absorption of cells without treatment of emodin as 100%. All determinations were made in triplicate

Bargmann *et al.*, 1986; Bargmann and Weinberg, 1988). The mutation-activated p185^{neu} in B104-1-1 is known to be associated with high tyrosine kinase activity. The transformation phenotypes associated with B104-1-1 cells are induced solely by the *HER-2/neu* oncogene. Therefore, the B104-1-1 cells served as an excellent model system to study the effects of emodin and DK-V-47 on the transformation phenotype induced by *HER-2/neu* oncogene. In addition, the phenotype induced by expression of mutated p185^{neu} has been reported to be similar to overexpression of normal p185^{neu} that also results in overall increase of tyrosine kinase activity. Therefore, the mutated p185^{neu}

has been used as a model to understand overexpression of normal p185^{neu}.

To address whether emodin and DK-V-47 can directly inhibit the tyrosine kinase activity of activated *HER-2/neu*, p185^{neu} was immunoprecipitated from the untreated B104-1-1 cells. The precipitates were then treated with varying concentrations of emodin and DK-V-47, and the kinase activity was measured, as shown in Figure 5, the tyrosine kinase activity for both autophosphorylation for p185^{neu} and transphosphorylation for enolase is inhibited by both emodin and DK-V-47 in a dose-dependent manner. DK-V-47 is more potent than emodin, a finding which is consistent with tyrosine phosphorylation status of p185^{neu} in breast cancer cells shown in Table 1. These results further demonstrate that both emodin and DK-V-47 directly inhibit the tyrosine kinase activity of p185^{neu} complex.

DK-V-47 is more potent than emodin in inhibiting both anchorage-dependent and -independent growth of B104-1-1 cells

Next, we examined the effects of the two tyrosine kinase inhibitors on cell growth rate. As shown in Figure 6a, the growth of *HER-2/neu* transformed 3T3 cells, B104-1-1 cells was inhibited by both emodin and DK-V-47 to varying degrees in a dose-dependent manner. At 80 μ M concentration, for example, emodin and DK-V-47 blocked 55% and 83% of the growth of B104-1-1 cells respectively. However, under the same condition, both emodin and DK-V-47 had little effect on the parental 3T3 cells.

These results indicate that emodin and DK-V-47 preferentially suppress growth of *HER-2/neu* transformed cells, and that DK-V-47 is more potent than emodin. They also suggest that the differential suppression effect occurs through the inhibition of the p185^{neu} tyrosine kinase activity, because the tyrosine kinase inhibitors have virtually no effect on the parental 3T3 cells. When the anchorage-independent growth activity of B104-1-1 cells was measured by a soft agarose colonization assay as shown in Figure 6b,

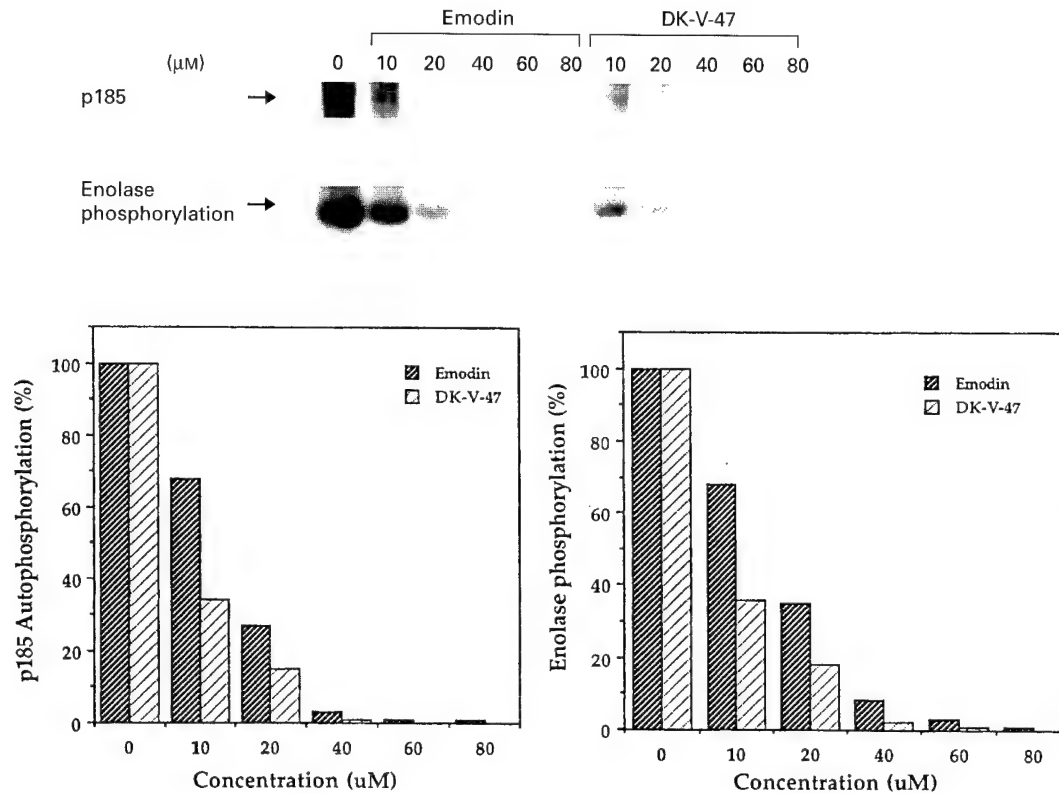


Figure 5 Emodin and DK-V-47 inhibit both autophosphorylation and transphosphorylation of p185^{neu} in activated *HER-2/neu* transformed 3T3 cells. Cell lysates from untreated cells were immunoprecipitated, then incubated with [γ -³²P]ATP, enolase and different concentrations of emodin and DK-V-47 at room temperature for 20 min. Reactants were resolved on 7.5% SDS-PAGE. The phosphorylation products were dried and visualized by autoradiography, as described in Materials and methods

DK-V-47 again behaved much more effectively in suppressing activity than did emodin.

Emodin and DK-V-47 suppress metastasis-associated properties induced by HER-2/neu oncogene

Tumor metastasis is a complex process involving a sequential series of critical steps (Liotta, 1986; Nicolson, 1988, 1991). The impartation of tumor cells in microcirculation and the subsequent invasion of blood vessel basement membrane are very important steps during blood-born metastasis. Gelatinase, type IV collagenases, has been shown to play a critical role in the dissolution of the basement membrane collagen during tumor cell invasion and metastasis (Nicolson, 1989). We have previously demonstrated that *HER-2/neu* transformed 3T3 cells can induce experimental metastasis in nude mice and enhance gelatinase activities (Yu and Hung, 1991). To determine whether or not emodin and DK-V-47 can decrease the activity of gelatinase collagenase IV in B104-1-1 cells, we examined the gelatinase activity in B104-1-1 cells with zymographic analysis. As shown in Figure 7, both DK-V-47 (A) and emodin (B) inhibit gelatinolytic activity of the 92 kDa and 68 kDa gelatinase, and this inhibition is enhanced with higher concentrations of both compounds. These results demonstrate that DK-V-47 is more effective than emodin; for example, at a

concentration of 10 μ M, DK-V-47 shows significant inhibitory activity for gelatinase, to achieve similar suppression, 40 μ M of emodin is required.

Cancer cell invasion is very important; to be metastatic, malignant cells in the blood must extravasate from the circulation, invade basement membrane and colonize distant sites. Activated *HER-2/neu* transformed 3T3 cells have been shown to be invasive (Yu and Hung, 1991). To determine whether or not emodin and DK-V-47 can abolish the invasive properties of activated *HER-2/neu* transformed cells, we performed *in vitro* invasion assay to monitor their effects. As shown in the Figure 8, both compounds can virtually abolish the ability of activated *HER-2/neu* transformed cells to penetrate the Matrigel layer. For repressing the invasive ability of *HER-2/neu* transformed cells, DK-V-47 is again more effective than emodin ($P < 0.0001$ at 25 μ M and $P < 0.05$ at 50 μ M).

Using the NIH3T3 cells transformed by the mutation-activated *HER-2/neu* oncogene, B104-1-1, as a model system, the results shown in Figures 5–8 clearly demonstrate that both emodin and DK-V-47 can inhibit the tyrosine kinase activity of p185^{neu} and thus abolish the transforming phenotypes induced by the *HER-2/neu* oncogene. These results support the conclusion that repression of p185^{neu} tyrosine kinase by these two compounds is probably the major mechanism which induces the preferential suppression of

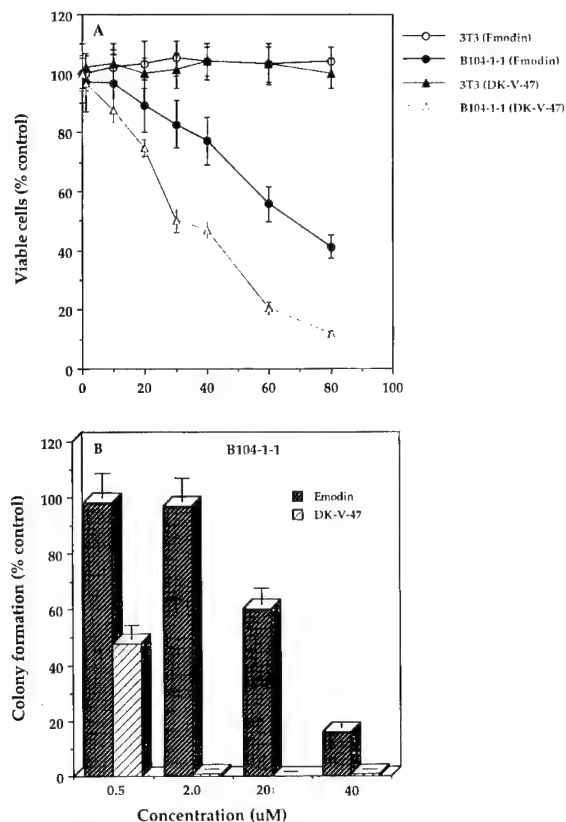


Figure 6 Emodin and DK-V-47 repress both anchorage-dependent and -independent growth of activated *HER-2 neu* transformed 3T3 cells. (a) Cells were incubated in the presence of serum along with or without different concentrations of emodin or DK-V-47 at 37°C for 72 h. The effect on cell growth was examined by an MTT assay, and the percentage of cell proliferation was calculated by defining the absorption of cells without treatment of emodin and DK-V-47, the controls, as 100%. All determinations were made three times; the results are means \pm s.d. (b) A soft agarose assay was performed as described in the Figure 2

biological activities for *HER-2/neu*-overexpressing cancer cells. The results also indicate that DK-V-47 is a much stronger suppressor than is emodin for *HER-2/neu*-induced transformation phenotypes.

Discussion

In our previous study (Zhang *et al.*, 1995), we demonstrated that emodin suppresses the tyrosine kinase activity of *HER-2/neu* and preferentially inhibits cell growth and the transformation phenotype *in vitro* for human breast cancer cells that overexpress the *HER-2/neu* oncogene. Furthermore, we found that emodin can sensitize *HER-2/neu* overexpressing non-small-cell lung cancer (NSCLC) cells to chemotherapeutic drugs such as cisplatin, doxorubicin and VP16, to which these cells are resistant (Zhang and Hung, 1996).

In the present study, we examined the relationship between the chemical structure and the inhibitory activity of emodin and its nine derivatives on the tyrosine phosphorylation of p185^{neu} and on the proliferation of human breast cancer cells. We

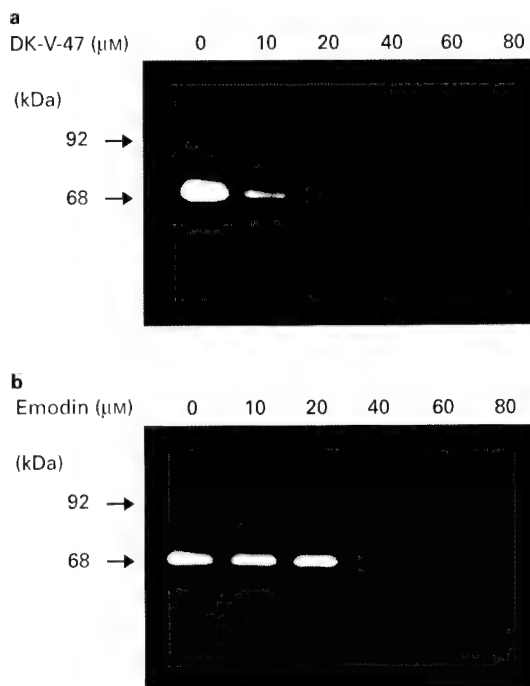


Figure 7 Emodin and DK-V-47 reduce gelatinolytic activity of activated *HER-2 neu*-transformed 3T3 cells. Cells were treated or not treated overnight with different concentrations of DK-V-47 (a) and emodin (b). The culture supernatants were then collected and analysed with zymography using gelatin-embedded SDS-PAGE. Gelatinolytic enzymes were detected as transparent bands on the blue background of Coomassie blue-stained gels, as shown by the positions of both the 92 kDa and the 68 kDa gelatinase

identified that one of the nine derivatives, DK-V-47, is more effective than emodin in repressing the tyrosine phosphorylation of p185^{neu} and in suppressing the transformation phenotype induced by the *HER-2/neu* oncogene. We have also found that emodin and DK-V-47 may arrest cell growth at G₀/G₁ phase by increasing numbers of cells in G₀/G₁ phase and reducing the numbers of cells in S-phase, under the same condition, we did not observe any apoptosis induced by treatment of either emodin or DK-V-47 (data not shown).

In our previous studies, we used human breast and lung cancer cells with different levels of *HER-2/neu* expression. It is well known that human cancer cell lines are associated with multiple genetic alterations, and *HER-2/neu* expression is not the only difference among the various human cancer cell lines. To investigate the specific effects of the tyrosine kinase inhibitors on the tyrosine kinase of p185^{neu}, in the present study, we used a *HER-2/neu*-transformed NIH3T3 cells line, B104-1-1, as a model. The results clearly indicate that DK-V-47 is a more potent suppressor than emodin in repressing the tyrosine kinase activity of p185^{neu} (Figure 5) and, accordingly, in transformation phenotypes induced by the *HER-2/neu* oncogene. In addition to differential activity to repress tyrosine kinase of p185^{neu}, we could not rule out the possibility that membrane permeability might also contribute to the different potencies between emodin and DK-V-47. Taken together, this study provides strong evidence that repression of the p185^{neu} tyrosine kinase activity by emodin and DK-V-47 plays a major

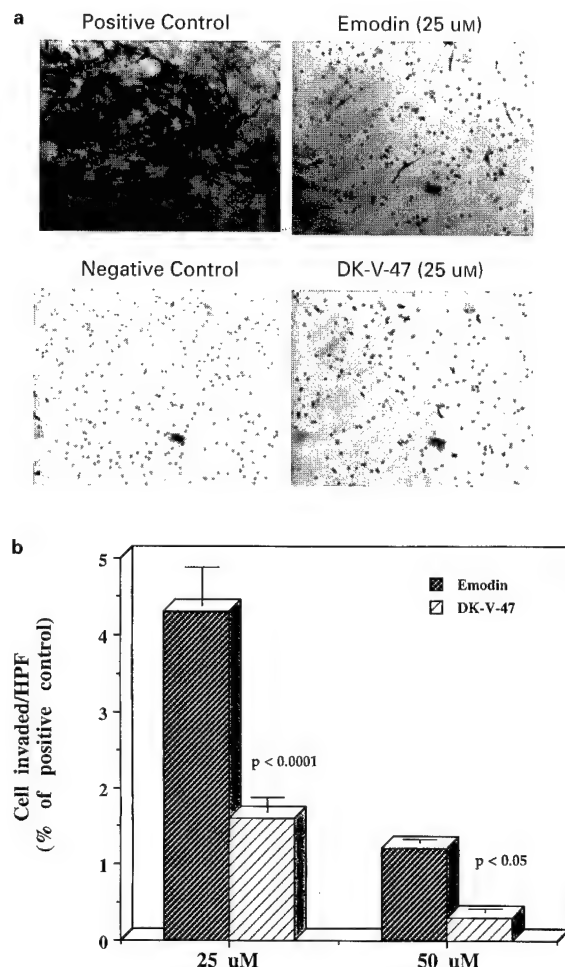


Figure 8 Emodin and DK-V-47 inhibit *in vitro* invasion of activated *HER-2/neu*-transformed 3T3 cells. *In vitro* invasion was measured by using 24-well Transwell units with an 8 mm pore polycarbonate filter coated with Matrigel to form a thin, continuous layer on the filter top. The lower compartment contained 0.6 ml of laminin (20 mg/ml) as a chemo-attractant. Activated *HER-2/neu*-transformed cells (5×10^4) were placed in the upper compartment and treated or not treated for three days with emodin or DK-V-47 in the presence of serum. The lower surfaces of the filters from the Transwell units were then fixed with 3% glutaraldehyde in PBS and stained with Giemsa solution (a). Chemo-invasive activity was determined under the microscope by counting cells that had migrated to the lower side of the filter (b). All experiments were done in triplicate; the results are means \pm s.d.

role in the preferential suppression effects of the *HER-2/neu*-overexpressing human cancer cells over the *HER-2/neu* basal level cancer cells.

With regard to the relationship between the structure of emodin and its nine derivatives and their inhibitory activity, the ten compounds were studied in four functional groups as shown in Table 1 and Figure 1. The first group is comprised of the compounds in which the CH_3 group at the C_3 position of emodin is replaced by one of four other functional groups. Their activities for the inhibition of the tyrosine phosphorylation of p185^{neu} and the proliferation of cells are dramatically reduced; suggesting that the CH_3 group at the C_3 position is critical for these activities. The OH group

at C_6 position of emodin is replaced by either H or OCH_3 group in the second group. These modifications also diminish the inhibitory activity as compared with emodin; this finding indicates that the OH group at the C_6 position is also critical for inhibitory activity. In the third group, the ketone group at C_{10} position of emodin is replaced by H group. Its inhibitory activities were significantly decreased as compared with emodin. These results indicate that the carbonyl group at C_{10} position is important for maintaining the inhibitory activity of emodin. The fourth group is comprised of the compounds in which the ketone at C_{10} position of emodin is replaced by the *p*-acetamidobenzylidene group. The compound DK-V-47 demonstrates more potent activity than does emodin. The CH_3CO group in the backbone of DK-V-47 appear to be critical for the biological activity; replacement of this group by H almost completely abolishes the biological activities. The hydroxyl group at C_6 , methyl group at C_3 and the ketone group at C_{10} are important for the biological activity of emodin as shown in Table 1. Interestingly, DK-V-47 which does not have C_6 hydroxyl and C_3 methyl groups is also associated with profound potency. The data suggest that multiple functional groups may contribute to the biological activities of emodin backbone, and a more systematic study is required to address the detailed structural and functional relationship.

Scholar and Toews (1994) reported that the tyrosine kinase inhibitor genistein can inhibit the invasion of murine mammary carcinoma cells; repression of tyrosine phosphorylation may be the mechanism. We have previously shown that both the mutation-activated *HER-2/neu* oncogene and overexpression of the normal *HER-2/neu* gene can enhance critical metastatic potential including the invasive ability to penetrate the basement membrane preparation Matrigel (Yu and Hung, 1991; Yu et al., 1994). Both mutation-activation and the overexpression of *HER-2/neu* result in increased tyrosine kinase activity. And as shown in Figures 5, 7 and 8, we have shown that both emodin and DK-V-47 can inhibit the tyrosine kinase activity of mutation-activated *HER-2/neu* in 3T3 cells, and both compounds can repress the secretion of gelatinase and invasive potential. These findings suggest that tyrosine kinase activity of *HER-2/neu* is required for the metastatic process, and that inhibitors of the tyrosine kinase activity of *HER-2/neu* such as emodin and DK-V-47 can repress metastasis induced by activated *HER-2/neu*.

In our preliminary study, we found that emodin can also repress EGF-induced tyrosine phosphorylation of EGF receptor at higher concentration, compared with the concentration which used to repress tyrosine phosphorylation of *HER-2/neu* (data not shown). It would be interesting to further study whether emodin or DK-V-47 might have differential selectivity in repression of activities of different tyrosine kinase molecules.

In summary, our results indicate that a tyrosine kinase inhibitor such as emodin or DK-V-47 which is capable of repressing the tyrosine kinase activity of *HER-2/neu* can effectively inhibit the transformation, and *in vitro* invasion of *HER-2/neu* overexpressing cancer cells. The stronger tyrosine kinase inhibitor DK-V-47 may serve as a better suppressor of *HER-2/neu*-mediated transformation than emodin. These results may have important chemotherapeutic implications.

Materials and methods

Preparation of emodin derivatives

All emodin derivatives were prepared from emodin using the synthetic procedures described previously (Kim *et al.*, in press). These compounds are stable under the cell culture condition, no decomposition was observed within 72 h at 37 °C.

Cell culture

Human breast cancer cells MDA-MB453, overexpressing *HER-2/neu* and MCF-7 cells expressing basal level of *HER-2/neu* were obtained from the American Type Culture Collection (Rockville, MD). The activated *HER-2/neu* transformed 3T3 cell line, B104-1-1, was established by transfecting the rat point mutation *neu* which possess high tyrosine kinase activity, into NIH3T3 cells (Hung *et al.*, 1989; Stern *et al.*, 1986; Bargmann and Weinberg, 1988). All cells were grown in DMEM/F12 (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum and gentamicin (50 µg/ml). Cells were grown in a humidified incubator at 37 °C under 5% CO₂ in the air.

Western blot analysis

Cells were treated by different concentrations of emodin and derivatives for 24 h in the absence of serum, then cells were washed three times with PBS and lysed in lysis buffer (20 mM Na₂PO₄, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% aprotinin, 1 mM phenylmethanesulfonyl fluoride, 10 mg/ml leupeptin, 100 mM NaF, 2 mM Na₃VO₄) as previously described (Zhang *et al.*, 1995). The protein content was determined against a standardized control using the Bio-Rad protein assay kit (Bio Rad Laboratories, Hercules, CA). A total of 50 µg of protein was separated by 6% SDS-PAGE and transferred to nitrocellulose filter paper (Schleicher & Schuell, Inc., Keene, NH). Nonspecific binding on the nitrocellulose filter paper was minimized with a blocking buffer containing non-fat dry milk (5%) and Tween 20 (0.1%, v/v) in PBS (PBS/Tween 20). The treated filter paper was incubated with primary antibodies (anti-p185^{neu} antibody c-*neu* [Ab-3] for the detection of p185^{neu} or with anti-phosphotyrosine antibody [UBI, Lake Placid, NY] for the detection of phosphotyrosine). This was followed by incubation with HRP-goat-anti-mouse antibody (1:1000 dilution) (Boehringer Mannheim Corp., Indianapolis, IN). Bands were then visualized with an enhanced chemiluminescence system (Amersham Corp., Arlington, IL) and quantitated using 'NIH Image' software.

Immuno-complex kinase assay

The immuno-complex kinase assay was modified from those described previously (Zhang *et al.*, 1995). Briefly, untreated cells were collected and then lysed in lysis buffer. Cell lysates (500 µg) were incubated with monoclonal anti-p185^{neu} antibody c-*neu* (Ab-3) for 1 h at 4 °C, then precipitated with 50 µl of protein-A-conjugated agarose (Boehringer Mannheim) for 30 min at 4 °C. The cells were then washed three times with 50 mM Tris-HCl buffer containing 0.5 M LiCl (pH 7.5) and once in an assay buffer (50 mM Tris-HCl, pH 7.5, 10 mM MnCl₂). To 40 µl of beads (protein-A-conjugated agarose), 10 µCi of [γ -³²P]ATP (Amersham) and 10 µl of enolase (Sigma Chemical Co., St. Louis, MO) and 1.0 µl of different concentrations of emodin and DK-V-47 were added and incubated at room temperature for 20 min. The reactants were separated by 7.5% SDS-PAGE. The gel was then dried, visualized by autoradiography and quantitated by 'NIH Image' software.

Cell proliferation by MTT assay

Cells were detached by trypsinization, seeded at 2×10^4 cells/ml in a 96-well microtiter plate overnight, and then treated with different concentrations of test samples of emodin and its derivatives and incubated for an additional 72 h in the presence of serum. The effects on cell growth were examined by MTT assay (Mosmann *et al.*, 1983; Rubinstein *et al.*, 1990). Briefly, 20 µl of MTT solution (5 mg/ml) (Sigma) was added to each well and incubated at 37 °C for 4 h. The supernatant was aspirated, and the MTT formazan formed by metabolically viable cells was dissolved in 150 µl of DMSO, and then monitored by a microplate reader (Dynatech MR 5000 fluorescence, Dynatech Corp., Burlington, MA) at a wavelength of 590 nm.

DNA content analysis

MDA-MB453 cells were treated with or without either emodin (40 µM) or DK-V-47 (20 µM) for 3 days in the presence of serum, then cells were collected by trypsinization and fixed for 30 min at -20 °C in 70% ethanol, 30% phosphate buffered saline (PBS) mixture. After staining with PBS containing 50 mg/ml propidium iodide and 8 µg/ml RNase A, cells were analysed on EPICS PROFILE flow cytometer (Coulter) as described (Kiyokawa *et al.*, 1997).

Colony formation in soft agarose

Cells were seeded in 24-well plates (1×10^5 cells/well) in culture medium containing 0.35% agarose (FMC Corp., Rockland, ME) over a 0.7% agarose layer and incubated for 4 weeks at 37 °C, as previously described (Zhang *et al.*, 1995). Colonies were then stained with p-iodonitrotetrazolium violet (1 mg/ml), and colonies larger than 100 µm were counted. Each measurement was made four times.

Zymography of gelatinolytic activity

As previously described (Yu and Hung, 1991; Yu *et al.*, 1994), cells were detached by trypsinization, seeded at 2×10^6 cells/well in a 6-well plate and cultured in DMEM/F12 medium supplemented with 1% FBS overnight, the cells were then washed with PBS and serum-free DMEM/F12 medium was added; then the cells were treated with different concentrations of test samples and incubated for an additional 24 h. The culture supernatants were collected, and then centrifuged at 800 g for 10 min, and then again at 18 000 g for 10 min. The supernatants (150 µl) were analysed by zymography using SDS PAGE containing 1.5% gelatin prepared according to procedures described previously (Yu and Hung, 1991; Yu *et al.*, 1994).

In vitro chemoinvasion assay

In vitro invasiveness was conducted according to the procedure described previously (Yu and Hung, 1991; Yu *et al.*, 1994), with modifications. Briefly, 24-well Transwell units with 8-mm pore size polycarbonate filter (Costar Corp., Cambridge, MA) were coated with 0.1 ml of a 1:30 dilution (48 mg filter) of Matrigel in cold DMEM/F12 medium. These filters were then air dried at room temperature, thus forming a continuous thin layer on top of the filter. The lower compartment contained 0.6 ml laminin (20 mg/ml, Becton Dickinson) as a chemoattractant or DMEM/F12 medium as a negative control. The cells (1×10^5 cells/0.1 ml of DMEM/F12 containing 0.1% bovine serum albumin) were placed in the upper compartment and incubated with or without either emodin or DK-V-47 at 37 °C for 72 h in a humidified atmosphere of 95% air, and 5% CO₂. Following incubation, the filters were fixed with 3% glutaraldehyde in PBS and stained with Giemsa. The number of cells per high-power ($\times 200$) field

that had migrated to the lower side of the filter were counted.

Statistical analysis

Statistical analysis was performed with student's *t* test.

Abbreviations

Abbreviations used are: DK-V-47, 10-(4-acetamidobenzylidene)-9-anthrone; DMEM/F12, Dulbecco's modified Eagle's medium/Ham's F-12; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel

electrophoresis; MTT, 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide; DMSO, dimethyl sulfoxide; IP, immunoprecipitation; WB, Western blotting; PY, phosphotyrosine; ATP, adenosine triphosphate.

Acknowledgements

This study was partially supported by grants from National Institute of Health CA 58880, CA 60856 (to MCH) and CA 50743 (to CJC), US Army (DAMD 17-94-J-4315 and 17-96-I-6253), Nelly Connally Breast Cancer Fund and National Research Institute of Chinese Medicine (to MCH).

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Safety studies of the intraperitoneal injection of E1A-liposome complex in mice

X Xing¹, V Liu¹, W Xia¹, LC Stephens², L Huang³, G Lopez-Berestein⁴ and M-C Hung¹

¹Department of Tumor Biology and Breast Cancer Basic Research Program, ²Department of Veterinary Medicine, and ³Section of Immunobiology and Drug Carriers, The University of Texas MD Anderson Cancer Center, Houston, TX; and ⁴Department of Pharmacology, University of Pittsburgh, Pittsburgh, PA, USA

The HER-2/neu (also called c-erbB-2) proto-oncogene is overexpressed in many human cancer cells, including those of breast cancer and ovarian cancer. We have previously shown that adenovirus 5 E1A inhibits HER-2/neu transcription and functions as a tumor suppressor gene in HER-2/neu-overexpressing cancer cells. Liposome-mediated E1A gene transfer suppresses tumor development and prolongs survival of tumor-bearing mice. In support of a phase I clinical trial of an E1A-liposome complex administered to patients with HER-2/neu-overexpressing breast or ovarian cancer, we conducted a series of studies to evaluate the safety of intraperitoneal injection of E1A in normal mice. The cumulative doses used were from five to

40 times the DNA-lipid starting dose proposed for the phase I clinical trial. In this dosing range, the administration of the E1A-liposome complex had no adverse effects on renal, hepatic and hematological parameters studied. No major organ pathologic changes were observed. We concluded that intraperitoneal administration of E1A-liposome complex at the proposed dose would not be expected to produce significant toxicity. The E1A-liposome clinical trial was recently approved by the Recombinant DNA Advisory Committee and Food and Drug Administration for a phase I trial in patients with HER-2/neu-overexpressing breast and ovarian cancer.

Keywords: toxicity; E1A-liposome; intraperitoneal injection

Introduction

Overexpression of the HER-2/neu (also called c-erbB-2) proto-oncogene occurs in many human cancer cells, including approximately 30% of breast cancer and ovarian cancer; and it is correlated with poor patient prognosis and shorter survival.^{1,2} Because down-regulation of HER-2/neu is able to reverse the transformation of cancer cells,³⁻⁷ HER-2/neu is considered an ideal target for cancer therapy.⁷⁻¹⁰ This concept is evidenced by our previous finding that adenovirus 5 E1A, a well-known transcription factor¹¹ and the early gene that is expressed after virus infection, inhibits HER-2/neu transcription as well as suppresses transformation and tumorigenicity induced by HER-2/neu-overexpression in cancer cells.^{3,12-14} Thus, E1A has great potential for use in gene therapy aiming at the HER-2/neu-overexpressing human cancers.

Cationic liposomes are the key to gene therapy studies as a nonviral vector. DNA-cationic liposome complex has been shown to efficiently transfect genes into a variety of tissues. It has advantages in several aspects compared with adenoviral and retroviral vectors: genes transduced by cationic liposomes are transiently expressed, less likely to be integrated into chromosomes, and are less likely to be able to allow propagation in cells. Lipids are

readily biodegradable once they are in contact with biological fluid or the intracellular environment. Thus, safety concerns related to the vector are minimized. In fact, studies from other groups have shown the low toxicity of DNA-liposome complex administration in mice,¹⁵⁻¹⁷ nonhuman primates^{18,19} and humans.¹⁶⁻²⁰ Repeated administration of DNA-liposome complex can be practically performed to reach higher gene transfer efficiency. Cationic liposomes are widely used in preclinical studies to deliver the genes of interest.²¹⁻²³

In our previous studies, we used the DOPE/DC-chole(dioleoyl-phosphatidylethanolamine 3b [N-(N,N'-dimethylaminoethane) carbamoyl] cholesterol) cationic liposome²⁴ to deliver the adenovirus E1A gene to mice bearing HER-2/neu-overexpressing human ovarian tumors.¹⁰ Expression of E1A and down-regulation of HER-2/neu-encoded p155 protein were detected in the tumor tissues, and approximately 70% of the treated animals survived for more than 1 year. These results suggest that cationic liposome-mediated E1A gene transfer may be a promising therapeutic strategy for HER-2/neu-overexpressing cancers.

Based on these preclinical studies, we planned to start a phase I clinical trial in patients with HER-2/neu-overexpressing breast and ovarian cancers using E1A-liposome complex. We conducted a series of studies to evaluate the safety of the intraperitoneal administration of E1A, using cumulative DNA and lipid doses equivalent to five to 40 times the proposed starting dose for the clinical trial in patients.

Table 1. Analysis of hepatic and renal functions in mice following a single dose of E1A-liposome complex by intraperitoneal administration

Group/Mouse No.	Dose (μg DNA/nmol lip)	Creatinine (mg/dl)	SCOT (IU/l)	
			Reading	Average \pm s.d.
1/1	7.5/100	0.5	90	110.6 \pm 16.15
1/2	7.5/100	0.5	116	
1/3	7.5/100	0.4	111	
1/4	7.5/100	0.4	138	
1/5	7.5/100	0.4	98	
2/1	15/200	0.4	102	125.2 \pm 42.38
2/2	15/200	0.4	112	
2/3	15/200	0.4	89	
2/4	15/200	0.4	208	
2/5	15/200	0.5	115	
3/1	30/400	0.5	70	106.2 \pm 46.67
3/2	30/400	0.5	127	
3/3	30/400	0.4	76	
3/4	30/400	0.5	189	
3/5	30/400	0.4	69	
4/1	60/800	0.4	165	107.2 \pm 30.46
4/2	60/800	0.4	92	
4/3	60/800	0.4	77	
4/4	60/800	0.4	110	
4/5	60/800	0.4	95	
5/1	120/1600	0.4	66	109.0 \pm 38.99
5/2	120/1600	0.4	90	
5/3	120/1600	0.5	115	
5/4	120/1600	0.4	171	
5/5*	120/1600	0.4	171	
6/1	150/2000	0.4	124	121.8 \pm 34.60
6/2	150/2000	0.4	113	
6/3	150/2000	0.5	182	
6/4	150/2000	0.4	76	
6/5	150/2000	0.4	114	

*Mouse died of internal bleeding at day 1.

Lip: DOPE/DC-chol liposome; s.d.: standard deviation.

Results and discussion

Acute toxicity study

To test the safety profile of the intraperitoneal injection of the E1A-liposome complex and to select the highest dosage for a later repeated-dose study,²⁵ six groups of mice were given single intraperitoneal injections of a wide range of E1A doses. The effective dose used in our previous preclinical gene therapy animal protocol was 15 μg ,^{4,10} which would be equivalent to the starting dose in our future phase I trial. In this experiment, each mouse received either 7.5 μg , 15 μg , 30 μg , 60 μg , 120 μg or 150 μg of E1A DNA. These doses were 0.5–10 times the starting dose proposed for our future phase I trial.²⁶ Control mice were injected with medium only. Mice were observed for clinical signs of toxicity daily, killed after 14 days, and analyzed for hepatic and renal function and gross pathology.

Major organ functions such as hepatic and renal functions were found to be normal in all appropriate groups by proper biochemical assays (Table 1). All organs had normal gross pathology (data not shown). Thus, this

series of intraperitoneal injections of E1A-liposome complex had neither lethal effects nor any signs of acute or residual toxic effects in mice over a 14-day observation period, even in the dosage as high as 10 times the proposed human dose. On the basis of these results, E1A dosages of 15, 30, 60 and 120 μg were chosen for the further repeat-dose study, i.e. after five consecutive daily injections, the cumulative doses are 5 to 40 times the proposed human dose for future phase I clinical trial.

Repeat-dose safety study

To assess further the safety of repeated doses of the E1A-liposome complex, five groups of five ICR mice each were treated intraperitoneally on a daily basis for 5 consecutive days, at an E1A dosage of 15, 30, 60 or 120 μg for each injection respectively, one group of mice were injected with medium as a negative control. No signs of clinical toxicity were observed at any of these dose levels during the 6-week observation period, and no E1A-related adverse effects were observed on the group body weight or group daily food consumption. Mice were

killed and necropsied for further analysis 6 weeks after the final injection.

Clinical chemistry and hematological analyses were performed on blood samples. The biochemical measurements of serum glutamic-oxaloacetic transaminase (SGOT) and blood creatinine are shown in Table 2. The SGOTs lay within the normal value range, suggesting that all these groups of mice had normal hepatic function. The creatinine assays gave biochemical values that reflect normal kidney functions. Blood counts are shown in Table 3 for the mice treated with the proposed dose of E1A-liposome complex for the proposed clinical study or the control reagent. The parameters indicate normal hematological values for these treated animals.

The safety profile of E1A-liposome was also assessed histopathologically. Brain, heart, liver, kidney, lung and spleen were examined. There were no significant microscopic lesions observed that correlated with E1A-liposome administration (Table 4). Focal invasion of lymphocytes, hemorrhage, and congestion found in the lungs were observed in some mice, however, these are most likely related to carbon dioxide poisoning which is used when killing the mice. Slight focal invasion of lymphocytes occurred in the kidney and liver, which is also a normal occurrence for this strain of mice.²⁷ In the spleens, three kinds of lymphoid hyperplasia and the formation of follicles with germinal centers in the marginal zones of the white pulp were observed. Most of these

alterations in the spleen did occur with greater frequency in treated animals; however, this is usually thought to be an incidental and nonspecific finding in this strain of mice. Thus, these changes were not deemed significant.²⁷ These data suggest that repeated-dose treatments with E1A-liposome did not result in significant lesions in the major organs tested; since cationic liposome induces transgene expression in cells transiently, observation of up to 6 weeks after injection is long enough to detect the adverse effects if there are any. Therefore, the proposed dosage administration of E1A-liposome complex in humans would not be expected to cause any organ pathology for patients.

Pathologic studies were also performed on mice at different time-points after a single injection at 10 times the proposed dose. The tissues were collected at selected time-points from several hours up to 14 days after injection. No biologically significant alterations in the tissues from three groups of mice were observed. In the remaining mice, alterations potentially attributable to E1A-liposome complex administration were generally minimal and highly localized (Table 5). Thus, our conclusion from these experiments is that the proposed human dose is a safe starting dose of E1A-liposome complex for the phase I clinical trial.

Similarly, other groups reported the lack of toxicity using the cationic liposome as a gene delivery system. The safety profile of plasmid DNA and DMR1E/DOPE

Table 2 Analysis of hepatic and renal functions in mice following five daily doses of E1A-liposome complex by intraperitoneal administration

Group/Mouse No.	Dose (μ g DNA/nmol lip)	Creatinine (mg/dl)	SGOT (IU/l)	
			Reading	Average \pm s.d.
1/1	0/0	0.3	165	118.0 \pm 36.63
1/2	0/0	0.4	116	
1/3	0/0	0.5	72	
1/4	0/0	0.4	84	
1/5	0/0	0.5	153	
2/1	15/200	0.5	210	135.4 \pm 58.27
2/2	15/200	0.4	196	
2/3	15/200	0.5	122	
2/4	15/200	0.4	83	
2/5	15/200	0.4	66	
3/1	30/400	0.4	129	114.0 \pm 24.28
3/2	30/400	0.3	129	
3/3	30/400	0.4	94	
3/4	30/400	0.4	77	
3/5	30/400	0.3	141	
4/1	60/800	0.5	200	149.8 \pm 38.21
4/2	60/800	0.5	151	
4/3	60/800	0.4	82	
4/4	60/800	0.4	163	
4/5	60/800	0.4	153	
5/1	120/1600	0.4	164	162.0 \pm 35.65
5/2	120/1600	0.4	149	
5/3	120/1600	0.4	223	
5/4	120/1600	0.5	147	
5/5	120/1600	0.4	122	

Lip: DOPE/DC-cholesterol liposome; s.d.: standard deviation.

Table 3 Hematological profiles in mice following five daily doses of E1A-liposome by intraperitoneal administration

Group/ Mouse No.	Dose	WBC ($10^3/\text{mm}^3$)	RBC ($10^6/\text{mm}^3$)	Hb (g/dl)	Hct (%)	MCV (fL)	Sg	Eosin	Mono	Lymph
1/1	0/0	6.8	8.68	15.0	38.2	44.0	9	1	0	88
1/2	0/0	8.7	9.09	15.7	39.8	43.0	17	2	0	80
1/3	0/0	6.4	8.43	15.4	38.8	46.0	18	1	0	91
1/4	0/0	—	—	—	—	—	—	—	—	—
1/5	0/0	4.4	7.70	14.1	36.3	47.8	32	2	0	65
2/1	15/200	5.1	8.71	14.8	37.9	43.0	13	2	0	85
2/2	15/200	11.6	7.61	13.1	32.9	43.0	9	2	0	89
2/3	15/200	9.9	8.39	14.8	38.2	46.0	15	1	1	83
2/4	15/200	3.7	9.44	16.8	40.1	42.0	15	4	0	61
2/5	15/200	—	—	—	—	—	—	—	—	—

*Data for mice numbers 1/4 and 2/5 are not available because the blood samples were coagulated due to a technical error. WBC, white blood cell; RBC, red blood cell; Hb, hemoglobin; MCV, mean corpuscular volume; Hct, hematocrit; Sg, segmented neutrophil; Eosin, eosinophil; Mono, monocyte; Lymph, lymphocyte.

Table 4 Histological analysis of mice following five daily doses of E1A-liposome by intraperitoneal administration

Group No.	Dose (μg DNA/ nmol liposome)	Histological lesions, number of mice with lesions/total mice
1	0/0	Mild lymphoid hyperplasia in the spleen of 1/5 mouse No lesions in 4/5 mice
2	15/200	Modest lymphoid hyperplasia in the spleen of 1/5 mouse No lesions in 4/5 mice
3	30/400	Mild lymphoid hyperplasia in the spleen of 2/5 mice and moderate in 1/5 mouse No lesions in 2/5 mice
4	60/800	Mild lymphoid hyperplasia in the spleen of 2/5 mice No lesions in 3/5 mice
5	120/1600	Moderate lymphoid hyperplasia in the spleen of 3/5 mice

liposome complex administration was evaluated in rodents and nonhuman primates by Parker *et al.*¹⁰ Compared with our studies, the liposome formulation is different, lower dosage lipid was tested, and intravenous and intrahepatic injection were performed. However, no significant adverse effects, no autoimmunity and organ toxicity were found to be associated with the DNA-liposome complex administration. In another study, patients with late stage melanoma were treated by intratumor injection with plasmid DNA encoding major histocompatibility complex HLA-B7 protein complexing with the DOPE/DC-chol liposome.¹³ The protein expression in tumor nodules was detected, and tumors were found regressive in some cases, but no toxicity and anti-DNA antibody was detected in any patients. Together with our safety studies reported here, these findings suggest that cationic liposome by several routes of administration is a gene delivery system with efficient gene transfer and the minimum adverse effects.

In light of the toxicity data reported here, the E1A-liposome phase I clinical trial protocol, in which the E1A-liposome complex will be injected into patients with HER-2/*neu*-overexpressing breast and ovarian cancers, has been approved by the Recombinant DNA Advisory Committee of the National Institutes of Health and the National Food and Drug Administration.

Materials and methods

Mice

ICR female mice, 4- to 6-weeks old, were purchased from Harlan Sprague Dawley (Indianapolis, IN, USA), and were cared for and used in accordance with institutional guidelines. Mice were housed for 1-2 weeks before treatment. Each mouse considered healthy was then injected with the E1A-liposome complex or an equal volume of Dulbecco's modified Eagle's medium (DMEM):F12 medium (1:1, GIBCO, Grand Island, NY, USA) serum-free medium under aseptic conditions.

Liposome-E1A complex

E1A DNA was prepared by bacterial fermentation and extracted using a Wizard DNA Maxi-preparation kit (Promega, Madison, WI, USA). DNA was precipitated with ethanol and resuspended in sterilized TE buffer. The cationic liposome was made in Dr Leaf Huang's laboratory in the University of Pittsburgh, by mixing DC-chol:DOPE in a molar ratio of 6:4.²⁴ The E1A-liposome complex was prepared under aseptic conditions on each day of dosing by diluting the proper amount of E1A-expressing vector in DMEM/F-12 serum-free medium and then mixing with the cationic liposome DC-chol:DOPE at a ratio of 15 μg DNA:200 nmol liposome to reach 200 μL . This mixture was then incubated at 37°C for 10 min before injection. DMEM/F-12 serum-free medium was used as a control.

Acute toxicity study

Thirty mice were separated into six groups of five mice each. Each group of mice received a different E1A-liposome dose: 7.5, 15, 30, 60, 120 or 150 μg in a single intraperitoneal injection. These doses were 0.5-10 times the proposed E1A-liposome complex starting dose for

Table 5 Histopathological analysis of mice at different time-points following E1A-liposome intraperitoneal administration

Group	Dose (μ g DNA/ μ mol lip)	Time of death	Histological lesions and number of mice/in total group with lesions
1	0/0	Predose	No lesions in 5/5 mice
2	150/2000	4 hours	No lesions in 5/5 mice
3	150/2000	1 day	Focal acute peritonitis, splenic-pancreatic mesentery in 1/5 mice Focal acute peritonitis-hepatic mesentery in 1/5 mice No lesions in 3/5 mice
4	150/2000	3 days	Hyperplasia sinus histiocytes of spleen in 5/5 mice Lymphoid hyperplasia of spleen in 1/5 mice EMH hyperplasia of spleen in 1/5 mice
5	150/2000	7 days	No lesions in 5/5 mice
6	150/2000	14 days	Hyperplasia sinus histiocytes of spleen with some nuclear debris in 1/5 mice Focal subacute peritonitis splenic-pancreatic mesentery No lesions in 3/5 mice

Lip, DOPE/DC-chol liposome; EMH, extramedullary hemopoiesis.

patients in our future phase I clinical trial.²⁴ Animals were observed for clinical signs of toxicity daily for the following 14 days and killed afterwards for analysis of hepatic and renal functions and for gross necropsy.

Repeat-dose toxicity study

Twenty-five female ICR mice were separated into five groups. Each group (except for the control group) of mice was given a different E1A-liposome dose (15, 30, 60 or 120 μ g, based on the acute study) daily for 5 consecutive days. The cumulative doses ranged from 5–40 times the proposed human dose for future phase I clinical trial. One more group of mice was injected with DMEM/F-12 serum-free medium as a control. Animals were observed for clinical signs of toxicity for the next 6 weeks. They were then killed for analysis of clinical chemistry, hematology, gross necropsy, and microscopic histopathology. Major tissues of these mice, including brain, heart, liver, lung, kidney and spleen, were fixed and preserved in 10% neutral buffered formalin and then embedded in paraffin. Sections of these tissues were stained with hematoxylin and eosin for further evaluation.

Acknowledgements

This research was in part supported by grants RO1-CA58880, RO1-CA60858 (to MCH) and RO1-CA64654 (to LH) and core grant 1b672 from NIH, and a contract from R Gene Therapeutics. Xiangming Xing is a recipient of the predoctoral fellowship from US Army breast cancer research program, Grant No. DAMA 17-96-1-6223.

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